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CONCEPTUALIZING AND IMPROVING RED WINE GRAPE CULTIVARS GROWN IN KENTUCKY

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Abstract

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Wine sensory attributes are associated with quality of wines. Cabernet Franc did not possess good coloration of its wine. Therefore, in the 2009 and 2010 growing seasons, studies including the sampling of four red wine grape cultivars from the end of flowering throughout the rest of the season and applying treatments to Cabernet Franc grapevines at veraison were commenced to address suitability and color enhancement, respectively. The study examining Cabernet Franc, Cabernet Sauvignon, Chambourcin, and Norton looked at sampling their grapes at two-week intervals from times post-flowering to understand the demands of each cultivar during key stages of berry development, in particular berry maturation post-veraison. The French-American hybrids Chambourcin and Norton were found to accumulate high levels of anthocyanins, also termed high cultivar performance, while the *Vitis vinifera* L. cultivars of Cabernet Franc and Cabernet Sauvignon remained stable in their anthocyanin content post-veraison. The results of the treatments applied to Cabernet Franc as a possible exogenous amelioration for anthocyanin pigment deficit in this cultivar support use of treatments for improving coloration in Cabernet Franc in Kentucky.

Key words: French-American hybrids, *Vitis vinifera* L., anthocyanins, treatments, organic acids

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THESIS

Matthew Davis Simson

The Graduate School

University of Kentucky

2011

CONCEPTUALIZING AND IMPROVING RED WINE GRAPE CULTIVARS GROWN IN KENTUCKY

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

2011

Dedicated to my parents, family, and friends who gave me the love, encouragement, and supported needed to accomplish this endeavor in my life.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

The compounds of interest in the research were phenolic compounds and organic acids, both play a vital role in winemaking and berry maturation. In wine, flavanols are antioxidants and vital to wine aging (Downey *et al.*, 2003b). Formation of polymeric pigments increases as wines age to represent a majority of coloration in wine (Harbertson *et al.*, 2003). It has been determined in *Vitis* species that genetic background plays a vital role in expression of anthocyanin coloration in the grape berry (Liang *et al.*, 2008). Fingerprinting of species and cultivars of a species, such as *Vitis vinifera* L., has allowed for models to accurately predict the cultivar based on its anthocyanin accumulation (Arozarena *et al.*, 2002; Ryan and Revilla, 2003). Organic acids provide the acidity required to prevent wine spoilage. Tartrate is inert to microbial spoilage and can be added to wines that are above their optimal pH range (Banhegyi and Loewus, 2004).

Wine phenolics play a tremendous role in the experience of tasting any wine. The sensory attributes of wine are renowned with its quality and thereby its success. Coloration of wine is associated with particular wine types and also wine varietals. Astringency is associated with the type of wine and the wine vintage. The justification of quantifying the phenolic composition of Cabernet Franc, Cabernet Sauvignon, Chambourcin, Merlot, and Norton wine varietals was to determine whether there was strong correlation between phenolic groups such as anthocyanins and tannins and ratings that were provided by a tasting panel sampling the wines that were quantified.

The phenolics and organic acids accumulation in grapes is a dynamic process involving the entire timespan of berry development. The best way of understanding how grape cultivars accumulate phenolic and anthocyanin compounds, both vital components in wine, is by observations at key times in grape development. A particular cultivar might be heat-sensitive, or become water-stressed easily (Kliewer and Torres, 1972). These climatic factors both affect the phenolic and organic acid accumulation ability of grapes. By looking at content of phenolics and organic acids in berries throughout the grape growing season, the trends of accumulation can provide distinction of grape cultivars. Being able to understand the demands that a cultivar has during berry maturation can

provide ways to improve quality associated with winemaking of red wine grapes in Kentucky.

Treatments affecting phenolic content are proposed as one way to overcome limitations that a cultivar might experience due to climate where it has been established. Such important properties in grapes that are worthwhile improving in the vineyard setting are grape coloration and phenolic content because these attributes are correlated with wine quality (Cheynier *et al.*, 1998). There are compounds which have been shown in previous study to improve coloration and phenolic content of grapes by enhancing or accelerating processes involved in berry maturation. By use of treatments enhancing or accelerating berry maturation the anthocyanin accumulation can be greater than with lesser maturing clusters. Such compounds with previous success in improving coloration and phenolic content and were part of our study are the compounds of ABA, benzothiadiazole, ethanol, ethephon, and the combination treatment of ethanol with ethephon have been used with the primary goal of improving coloration in the Cabernet Franc cultivar of *Vitis vinifera*.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Wines are complex in the nature of their composition. One class of compounds that has been looked at extensively in wines is phenolic compounds. These consist of the primary groups of catechins, anthocyanins, tannins, and polymeric pigments. Catechins are subunits which polymerize to form tannins (Thorngate, 1997). Catechins are also cofactors in association with anthocyanins to form copigments which augment the coloration of wine (Timberlake and Bridle, 1976). Anthocyanins are the compounds in wine that are associated with coloration. The forms of anthocyanin found in grapes are malvidin, delphinidin, petunidin, peonidin, and cyanidin (Hebrero *et al.,* 1989). The relative abundance of each form differs based on cultivar and climate (Brossaud *et al.,* 1999, Arozarena *et al.,* 2002). Anthocyanins are the primary source of coloration in new wines (Leone *et al.,* 1984; Gao *et al.,* 1997). As wines age, there is formation of polymeric pigments which replace anthocyanins as the major source of coloration (Somers, 1971; Harbertson *et al.,* 2003). Polymeric pigments are composed of anthocyanins and flavonol groups. Depending on the size of the flavonol group associating with the anthocyanin, the polymeric pigment will be classified as a small polymeric pigment or a large polymeric pigment (Harbertson *et al.,* 2003). Small polymeric pigments are composed of either the flavonols catechin or epicatechin, or of oligomeric proanthocyanidins; both flavanols and oligomeric proanthocyanidin are subunits of polymerized tannins. Large polymeric pigments are composed of anthocyanins associated with polymerized tannins.

Grapevine accumulation of phenolic compounds and organic acids is a process which is dictated by grape berry development. Early stages of berry development are marked by the accumulation of astringent tannins which act as feeding deterrents to herbivores (Lu and Bennick, 1998). Berry acidity is also high in immature grapes due to accumulation of the organic acids tartrate and malate from flowering through veraison where they are at maximum levels (Conde *et al.,* 2007). Veraison is the stage of the berry development that is noted by the onset of coloration of grape berries due to the accumulation of anthocyanins in the skins of red grapes. The increase in size of the grape berry at

veraison due to its accumulation of solutes such as sugars also leads to a decline in the concentrations of compounds like tannins and a reduction in the overall acidity of the berry which helps to encourage berry dispersal with more appealing and pleasant tasting berries at their maturity (Downey *et al.,* 2003a).

Phenolic compounds and organic acids contained in grapes are critical for winemaking. Wine flavonols are known to have an antioxidant nature and aid in the aging of wines (Downey *et al.,* 2003b). The formation of polymeric pigments increases as wines age to eventually represent the majority of the coloration of wines (Harbertson *et al.,* 2003). The complete conversion process of anthocyanin monomers to polymeric pigments can take place within three years (Somers, 1971). Organic acids are extremely important in providing the acidity that wine needs in order to not spoil. Tartrate has the property of being inert to microbial spoilage and it can be added if the wine pH is higher than is optimal (Banhegyi and Loewus, 2004). Other organic acids are known to be metabolized by wine microbes and are reduced. An example of reduction of an organic acid in wine is malolactic conversion of malate to lactic acid by bacteria that have been introduced to the wine (Main *et al.,* 2007).

The importance of phenolic compounds in creating wine has led to the use of treatments for grapes to alter the levels of phenolic compounds in grape berries. The treatments include plant hormones such as ABA (Yamane *et al.,* 2006). Treatments affect plant hormone levels in the case of ethephon and ethanol (Farag *et al.,* 1992). Treatments can also consist of compounds which induce systemic acquired resistance in the case of benzothiadiazole (Kunz *et al.,* 1997). The effect which is observed by treatment is an increase in the expression of enzyme genes that control the production of phenolic compounds by the phenylpropanoid pathway. Treatments using these compounds have increased coloration in the grape berry which means that expression of enzyme genes responsible for the production of anthocyanins, a group of phenolic compounds, has increased.

2.2 Wine Phenolics

2.2.1 Overview of phenolic compounds found in wine

The way a sip of wine is perceived by a taster is due in large part to the phenolic content of the wine. Flavonoids which compose the majority of wine phenolics are thought to be UV protectants and are known to have antioxidant behavior (Downey *et al.,* 2003b). Intended to be a feeding deterrent to herbivores through their bitter and astringent nature, some animals such as humans have developed ways to counter health-harming effects of phenolic compounds. One such mechanism is the presence of salivary proline-rich proteins (PRPs) which are highly effective at creating insoluble bonding with tannins (Lu and Bennick, 1998). Tannins have significant gustatory affect with their astringent and bitter nature (Thorngate and Noble, 1995; Thorngate, 1997). Although tannins in grapes are both bitter and astringent, a small amount of tannin in wine is associated with quality of the wine (Cheynier *et al.,* 1998). The flavan-3-ols catechin and epicatechin form condensed tannins as polymers and are also referred to as proanthocyanidins, contribute to body and mouthfeel properties of wine. Additionally, flavan-3-ols are rated as being bitter when tasted at the threshold of 20 mg/L in water (Thorngate, 1997). Soft tannins describe a wine that lacks bitterness but can also describe a wine that has undergone oxidative polymerization of flavanols (Cheynier *et al.,* 1998). When flavan-3-ols and their polymers are in high concentration in wine the sensation in the mouth is one of continuous drying of the tongue and palate due to their astringency (Thorngate, 1997). The reason for wine bitterness is still poorly understood due to the diversity of compounds responsible for bitter gustatory affect (Cortell *et al.,* 2008). Determining the actual tasting mechanism of flavonoids in wine is still an ongoing process and is made more difficult by the variability in sense of taste which exists in the human population (Thorngate, 1997).

Anthocyanins are what impart color to young red wine and grapes (Somers, 1971). Anthocyanins associate with flavonols; while flavonols themselves are colorless, their association as anthocyanin copigments influences wine color. In copigmentation of anthocyanins with flavonols, the monomeric flavonols are referred to as co-factors and

their proportion in relation to the amount of copigments found in the wine is suggested to influence wine coloration. As the concentration of anthocyanin increases or more cofactor is present, there is an increase in the blueness of the wine which is more dark and intense than the red that individual anthocyanins impart. The degree of blueness that is attributed to cofactors and anthocyanin formation of copigments has also been found to be due to the flavonol present. For example, the flavonol quercetin provides a large degree of color enhancement and catechin is considered to be much less strong in color enhancement (Boulton, 2001). With the addition of catechins and proanthocyanidins to malvidin glucoside solutions, the color augmentation of malvidin glucosides was observed to increase as the content of this form of anthocyanin and phenolics in the solution was increased (Timberlake and Bridle, 1976).

Anthocyanins also associate with tannins in polymeric complexes which become more prevalent when compared to individual anthocyanins as wine ages (Somers, 1971; Timberlake and Bridle, 1976; Vidal *et al.,* 2002). Polymeric pigments are considered to be either large polymeric pigments (LPP) or small polymeric pigments (SPP) depending on size of flavan-3-ol polymer associated with the anthocyanin. An example of a large polymeric pigment is an anthocyanin interacting with condensed tannins found in the skins or seeds and would precipitate with protein in a protein solution while an example of a small polymeric pigment is a catechin monomer linked to malvidin-3-glucoside and would not precipitate in a protein solution (Harbertson *et al.,* 2003). In the aging of wine in wooden barrels, particularly new barrels, hydrolysable tannins are released from the wood, but they are not flavonoid compounds. Hydrolysable tannins, also called ellagitannins, have a phenolic structure, being composed of hexahydroxydiphenic acid esters with glucose; their interactions in wine has impact on wine aging in processes like wine oxidation through limiting oxidation of wine flavonoid compounds (Vivas and Glories, 1996). Vivas and Glories (1996) observed ellagic tannins protecting the flavan-3-ol catechin from oxidation and noted a deeper hue in the wine having addition of ellagic tannins presumably due to less oxidation of phenolic compounds.

2.2.2 Anthocyanin content in wines

Anthocyanins increased in the initial stages of fermentation where they reached maximum concentration two to three days after beginning fermentation (Mazza *et al.*, 1999). The attainment of maximum levels of anthocyanin in wines two to three days after fermentation began was found previously by Leone *et al.* (1984). The evolution of anthocyanins existing in monomer form to being primarily contained in the wine as polymers is rapid. In fact, from the time when anthocyanins were at maximum level at the start of fermentation until bottling, anthocyanins existing as anthocyanin monomers declined substantially to the point that at bottling they were a small fraction of the maximum levels observed during the winemaking process (Gao *et al.*, 1997). Other sources have noted that wines lose the majority of their anthocyanin monomer content within a three year timespan. Evidence for such loss has been indicated by the percentage of anthocyanin polymers in the wine (Somers, 1971). Measurement of polymeric pigments has concluded an alteration in the manner in which anthocyanins function in aging wines by the amount of color which polymeric pigments impart to the wines versus the coloration that has been attributed to the monomeric anthocyanins; this conversion process was found to be most significant over a timespan of a few years (Harbertson *et al.*, 2003).

The effect of aging wines in stainless steel tanks versus oak barrels on anthocyanin content of the wine is something which could be significant in consumer perception of the wine by its appearance. An investigation of the effect of aging in oak barrels versus much larger stainless steel tanks revealed amounts of anthocyanins that were very similar in wines of either aging vessel (Revilla *et al.*, 2001).

The ability of the yeasts that were used in fermentation as a way of adsorbing anthocyanins has been indicated. The ability of yeast lees obtained from chardonnay wine to remove anthocyanins in a wine solution has been noted. In the study of Vasserot *et al.* (1997), the yeast lees utilized were found to remove an approximate 40% of the total anthocyanins within minutes of introduction which would allow champagne containing higher anthocyanin content to be reduced to a total content more comparable

to champagne having the lower anthocyanin content desired in champagne. The conclusion that was reached in the study by Vasserot *et al.* (1997) was that yeast lees are not as affective as charcoal to remove anthocyanins as they were found to be influenced by other properties of the wine solution.

Regarding the yeasts used in fermentation, non-significant effects on coloration of wines based on the type of yeast and wine varietal have been found. In the study by Mazza *et al.* (1999), only one yeast treatment for Pinot Noir was different for anthocyanin content of wine made from that yeast treatment and there were no significant differences for treatments done to Cabernet Franc and Merlot wines.

Microoxygenation was shown to affect anthocyanins and wine color. It was found that there were decreased monomeric anthocyanin levels in wines that had undergone microoxygenation. The microoxygenated wines had greater blue tones than the wines that were not microoxygenated (Perez-Magarino *et al.*, 2007). The enhanced blueness of microoxygenated wines suggested a higher level of copigmentation because blueness attributed to copigments has been described by Boulton (2001).

2.2.3 Analysis of flavonoids in wines and grapes

The use of analytical procedures to measure phenolics in wines and grapes is quite extensive. A great amount of research has dealt with measurement of tannins due to their high impact on a wine's overall taste. A protein precipitation method was developed as a way of quantifying the levels of tannins in grains, but now the use of protein to precipitate tannins is used in grape and wine analysis (Hagerman and Butler, 1978). Hagerman and Butler (1978) also determined that the protein precipitation method could be conducted at room temperature versus at a colder temperature which had previously been thought necessary due to fact that protein degrades at higher temperatures. Protein precipitation, phloroglucinolysis, and gel permeation chromatography have all been utilized in measurement of tannins found in wine (Kennedy *et al.,* 2006). Studies examining data results of tannin measurement have shown poor correlation of tannin content to the wine grape cultivar and this suggests that tannin measurements, regardless of method utilized, should be more of a qualitative tool to go along with wine tasting

panel astringency ratings versus having the quantitative measurement for the wine tannin being the sole criteria for astringency rating (Kennedy *et al.,* 2006; Harbertson *et al.,* 2008). Additionally, there are recent methods such as the modified Adam's assay and modified Glories' method which measure primary groups of wine phenolics and can allow better interpretation of the individual wine than procedures which only account for tannins (Mazza *et al.,* 1999; Harbertson *et al.,* 2003; Landon *et al.,* 2008).

2.2.4 Wine and grape antioxidant activity attributed to phenolic compounds

When accessing the antioxidant activity in both wine and grapes, correlations were made between content of individual phenolics and also overall phenolic content. Correlations of phenolics with LDL antioxidant activity were done and it was observed that gallic acid and catechin were both strongly correlated with LDL antioxidant activity while the anthocyanins measured showed only moderate correlation. Due to such diverse composition of wine of the various phenolics, it is likely that both positive and negative attributes exist regarding the affect of phenolics on their level of antioxidant activity. Furthermore, wines different in such complex compositon might exhibit characteristic behavior regarding their antioxidant potential (Frankel *et al.,* 1995).

Evidence of there not being a complete synergy of the phenolics contained in a wine on the level of antioxidant activity was noticed when wines containing particular phenol concentrations were compared to a model wine of the same phenol concentration containing only catechin as its phenolic constituent (Meyer *et al.,* 1997). The results of Meyer *et al.* (1997) were that the reference model wine composed of only pure catechin performed at a higher antioxidant activity than the wines which contain a complex mixture of phenols. Because wine compounds appearing in significant quantity, such as catechin and anthocyanin, appear to have the most significance in LDL antioxidant activity, it is considered to be unlikely that a compound such as the non-flavonoid resveratrol appearing in concentrations of only 1-2 mg per liter of red wine and approximately 2% of the resveratrol content of red wines is found in white wine has as equal or greater antioxidant activity as more abundant wine phenolics (Frankel *et al.,* 1995). The fact that the wine phenolic resveratrol exists in such small quantities in wine

suggests that it would have insignificant consequence on LDL antioxidant activity and cardiovascular health in general unless it were to have different means of activity than other wine phenolics.

The manner in which wines were made was investigated to determine how certain processes altered antioxidant activity. The flavonol compound flavan-3-ol, also known as catechin, was shown to be strongly correlated with LDL antioxidant activity in the review by Frankel *et al.* (1995), but was only present in significant quantity at 99 mg per liter in Petite Sirah only after an extraction of 165 hours (Meyer *et al.,* 1997). In the study of Meyer *et al.* (1997), it was found that the LDL antioxidant activity was highly correlated with the level of catechin in the wine extracts which gives solid support to the recommendation of extending maceration time as a way of increasing the LDL antioxidant activity of the wine.

However, the most important finding is that antioxidant activity has been shown to be based on correlation to many wine phenolics, so the most important aspect of a wine's overall antioxidant activity is due to it having an abundant amount of phenolics (Frankel *et al.,* 1995). This helps to explain that dark wines containing more anthocyanins when young have potentially greater antioxidant activity due to having high content of at least one of the primary wine phenolics, anthocyanins, and this is still being investigated (Munoz-Espada *et al.,* 2004). Establishing whether particular wine compounds exhibit synergistic or antagonistic affects on antioxidant activity can allow for more logical, scientific-based decisions on how to establish wine processes for wines having the highest expected antioxidant activity (Meyer *et al.,* 1997).

2.2.5 Management practices influencing berry flavonoid accumulation

In Pinot Noir vines, average size of tannin polymers was observed to increase as vine vigor decreased (Cortell *et al.,* 2008). There were higher levels of proanthocyanidins in the low-vigor vines which would mean that wine made from low-vigor vines has potentially greater astringency (Downey *et al.,* 2003a). However, due to grapes of low-vigor vines being higher in soluble solids at harvest, the wine made from them has higher alcohol content and this can be perceived as tasting bitter which might counteract low-

vigor being associated primarily as having greater astringency (Cortell *et al.,* 2008). While vigor is widely known to be cultivar dependent, there are management practices such as percentage of cluster exposed, the degree of pruning of the vine compared to harvest mass of grapes, and vine density per hectare, these practices are known to influence the phenolic compound levels and anthocyanin levels in grapes (Jackson and Lombard, 1993). The impact that stresses to the grapevine, such as heavy pruning and high percentage of cluster exposure, have on flavonoid accumulation is believed to be an increased biosynthesis of secondary metabolities of the flavonoid biosynthetic pathway to protect the plant from UV exposure and pathogens (Koes *et al.,* 1994).

In comparing non-irrigated versus irrigated vines, it was found that non-irrigated vines were less vigorous growing and had smaller grape clusters having greater concentrations of phenolics and anthocyanins, but that phenolic content of irrigated grapes was greater due to increased berry size and there was no significance in phenolic content based on the irrigation treatment at some of the sampling dates (Esteban *et al.,* 2001). Irrigation method: double normal irrigation, normal irrigation, and irrigation just above plant wilting point, all showed no significant difference in the ability to accumulate tannin or anthocyanin in the grape berry (Kennedy *et al.,* 2000). However, it has also been found that less irrigation results in an increase in anthocyanin concentration (Kennedy *et al.,* 2002). But, the intentional utilization of water deficit in a vineyard would not benefit flavanoid composition of the grapes for high quality wine use. It was found that there was no significant change in the degree of polymerization of proanthocyanidins, and the flavan-3-ol content of the grapes declined (Kennedy *et al.,* 2000). However, a greater anthocyanin to flavonol ratio in a minimally irrigated system than one which has received double normal irrigation supports copigmentation of anthocyanins with flavonols in the wine solution; greater copigmentation would contribute to the observation of greater flavonol concentration in wines (Kennedy *et al.,* 2002). Another drawback of minimal irrigation, or likewise, deficit irrigation, is that in some instances the plant stress during water deficit can alter the source-sink relationship of the grapevine and also lead to dessication of tissues such as berry epidermal tissue (Coombe, 1987).

Nitrogen fertilizer use has also been implicated in reduced anthocyanin accumulation in grapes. However, one study which varied soil composition substantially through the alteration of soil pH from the natural level of slightly acidic pH of 6.1 to an alkaline pH 8.0 for the soil treatment consisting of limestone soil and neutral pH 7.0 for the oyster shell soil treatment observed no significant difference in the amount of nitrogen contained in any of the treatments examined over two years (Yokotsuka *et al.*, 1999). This finding of non-significant difference in nitrogen levels suggests that nitrogen itself is not the reason for an observation of altered anthocyanin accumulation due to soil composition when the rate of nitrogen usage and nitrogen retention appears to be the same in all treatments.

2.2.6 Environmental influence on berry flavonoid accumulation

Location plays a role in the berry phenolic levels. When analyzing south and north Okanagan wines, differences in both color and astringency were noted and the conclusion was that the differences in terroir caused for these noted effects (Cliff *et al.*, 2007). Tannin levels are indeed influenced by growing site location in contrast to anthocyanins which were found to vary significantly from year to year independent of location; location in a study by Brossaud *et al.* (1999) referred to ten experimental plots in Loire Valley, France.

Being that location also determines the range of temperatures experienced in a growing season, studies looking at controlled day and night temperatures can serve to explain preference or lack of preference of a given cultivar for a particular growing region. Looking at several combinations of daytime and nighttime temperatures in the 15°C to 35°C range, an analysis of the anthocyanin pigmentation showed a preference of grapevines in their anthocyanin biosynthesis towards cool nights and daytime temperatures less than 30°C (Kliewer and Torres, 1972). Cool growing seasons have allowed greater anthocyanin accumulation as found in the study by Yokotsuka *et al.* (1999) which observed greater anthocyanin accumulation in three different soil treatments when compared to the previous growing season that was warmer.

In the study by Kliewer and Torres (1972), a linkage between cultivar performance defined as the ability to accumulate anthocyanin and the level of anthocyanin pigmentation usually found in berries of that cultivar was found with the cultivars Cabernet Sauvignon and Pinot Noir containing higher anthocyanin levels performing better than the Tokay cultivar containing lower levels of anthocyanin when grown in a high temperature environment.

2.3 Grape Berry Phenolics

2.3.1 Berry accumulation of flavonoids found in wine

Anthocyanins have been studied the most of all phenolic compounds in grapes because of the dynamic behavior they have during biosynthesis in grape berries on the vine and while wine ages. Anthocyanins have been found to be at their highest concentration 20 to 25 days after veraison but not highest in overall content due to berry size not being at its maximum (Mazza *et al.,* 1999). This trend in anthocyanin concentration being highest roughly three weeks after veraison has also been observed in Somers (1976) for grapes grown in South Australia and Roggero *et al.* (1986) for grapes grown in Cotes du Rhone. Their overall content is highest at harvest and two to three days after the start fermentation (Mazza *et al.,* 1999). For mature grapes, the content of anthocyanin is estimated to range from 200 mg/kg to 5,000 mg/kg of fresh grape. The principal form of anthocyanin at harvest time is malvidin and is what provides the characteristic deep red color associated with young wines (Jackson, 2008). The grapes used to study the anthocyanin composition of grapes and their antioxidant activity are generally from the species *Vitis vinifera* L. (Frankel *et al.,* 1995; Meyer *et al.,* 1997; Serafini *et al.,* 1998; Mazza *et al.,* 1999). When Concord grapes were analyzed for their anthocyanin composition, over 40% of their total anthocyanin was of the delphinidin form of anthocyanin and this was attributed to the fact that Concord is a cultivar from the *Vitis labrusca* L. species of grape (Liang *et al.,* 2008). One cultivar grown in the Eastern U.S. is Norton and is a cultivar of the *Vitis aestivalis* Michx. species of grape; it is hypothesized that it and other grape hybrids would contain different amounts of

anthocyanins. When compared to Foch and Concord, two other non-*Vitis Vinifera* grapes, Norton produced significantly more anthocyanins in its skin and in wine made from the cultivar, which attests to the affect that genetic diversity based on species of grape can have on the amount of anthocyanin produced in grapes (Munoz-Espada *et al.,* 2004).

A predominance of research has looked at anthocyanin biosynthesis in the field of flavonoid biosynthesis perhaps due to the fact that anthocyanins possess coloration (Boss *et al.,* 1996a). Research has also looked at flavonol biosynthesis in red and white grapes (Downey *et al.,* 2003b). The distribution and accumulation of tannin in the grape berry is well-known (Coombe, 1987). Tannin levels in the skin accumulate from flowering until two weeks after veraison, while in the seeds the accumulation begins immediately after fruit set and reaches a maximum at veraison (Downey *et al.,* 2003a). The condensed tannins observed in skin and seeds are different in their subunits. Skin tannins are longer polymers with mean degree of polymerization (mDP), or molar ratio of total units to end units, being of approximately 36 units and skin tannins possess mostly epicatechin subunits. Seed tannins have a more equal balance of catechin and epicatechin subunits and are considered short polymers having an mDP of only 9.5 compared to the mDP of 36 observed for skin tannins (Vidal *et al.,* 2002). The majority of epicatechin subunits explains the astringent nature skin tannins impart to wine, while seed tannins have greater bitterness due to a more equal balance of catechin and epicatechin subunits (Thorngate and Noble, 1995; Cheynier *et al.,* 1998). Decreasing extractability of tannins is noted as the berry approaches harvest and coincides with the dispersal strategy of the grapevine which includes sugar accumulation and anthocyanin biosynthesis in the grape berries (Downey *et al.,* 2003a).

Difference in the accumulation of subunits composing berry tannins has been found in regards to relative percentages, but amount of tannins were not significantly different (Brossaud *et al.,* 1999). The observation of tannin amount in grapes remaining relatively unchanged could explain the rationale behind classification of wines based on astringency due to the fact that repeatable tiers of astringency exist from year to year for white, red, and full-red wines (Landon *et al.,* 2008).

Anthocyanin biosynthesis begins approximately 10 weeks post-flowering and expression of the genes involved in anthocyanin biosynthesis is very apparent for up to 4 weeks and again weeks 8 to 10 at veraison. Similar gene expression, including UDP glucose-flavonoid 3-0-glucosyl transferase (UFGT) resumes at veraison where it is believed that UFGT is responsible for turning on the genes associated with anthocyanin biosynthesis and coincides with anthocyanin accumulation in the berry (Boss *et al.,* 1996a). It was further presumed that there are two regulatory genes involved in the berry skin with one present at flowering and turning on all structural genes except UFGT and then a second regulatory gene connected to turning on UFGT but it is not certain whether both regulatory genes are present concurrently. There are two branches associated with synthesis of different forms of anthocyanins in grape berries with one branch containing Cyanidin-3-glucoside and by methyltransferase to Peonidin-3-glucoside and the other branch producing Delphinidin-3-glucoside which by methyltransferase forms Petunidin-3-glucoside and Malvidin-3-glucoside; it was found both branches have a nearly constant rate of flux through ripening (Boss *et al.,* 1996a). In mature Cabernet Sauvignon grapes Malvidin-3-glucoside was found to be the primary anthocyanin conformation at roughly 46% of total anthocyanin (Revilla *et al.,* 1998). In fact, for six cultivars studied, Malvidin-3-glucoside was more prevalent than any other form of anthocyanin (Revilla *et al.,* 2001). Malvidin-3-O-glucoside was found to be the major anthocyanin conformation in grapes in earlier studies, as well (Bakker and Timberlake 1985, Roggero *et al.,* 1986).

Separation of grape species can be achieved by utilizing the fact that for anthocyanins found in *Vitis vinifera*, only the five monoglucosides malvidin, delphinidin, petunidin, peonidin, and cyanidin are in grapes while anthocyanin diglucosides exist in other grape species (Hebrero *et al.,* 1989). One variety, Pinot Noir, only contains unacylated forms of the anthocyanins, but in other varieties of *Vitis Vinifera*, anthocyanins appear partially acylated (Mazza and Francis, 1995; Gao *et al.,* 1997). At a time 20 days after veraison until the harvest date, it was observed that the relative ratio of the five monoglucosides found in grapes remained unchanged with only the quantity of the anthocyanins changing (Arozarena *et al.,* 2002). Previous studies confirm the fact that it is quantity of the forms of anthocyanin and not the relative percentage of total anthocyanin which changes as grapes mature (Yokotsuka *et al.,* 1999; Brossaud *et al.,* 1999). When looking at

anthocyanin accumulation in Cabernet Franc berries, very insignificant changes in percentages of the top three anthocyanins ($p < 0.05$) were observed from the first and the second year of the study which suggests underlying genetic factors contribute to form of anthocyanin accumulated and the percentage of each anthocyanin form was found to be relatively stable in accumulation during berry ripening (Brossaud *et al.,* 1999). The percentage of unacylated glucosides in a grape cultivar was found to remain unchanged from year to year when analyzing three grape cultivars and that when percentage of the primary monoglucoside Malvidin-3-glucoside changed that the percentage of the other unacylated monoglucosides adjusted accordingly to arrive at a repeatable percentage of the total anthocyanin for a given cultivar (Arozarena *et al.,* 2002).

While some variability exists in the percentage composition of anthocyanin monoglucosides within a given cultivar, the data of Arozarena *et al.* (2002) was effectively utilized in a model which correctly predicted grape variety and only failed to assign classification of the correct vineyard. Another study looking at prediction of cultivar using climate and percentages of three anthocyanin conformations to correctly predict cultivars of all of the samples and only incorrectly identified the climate as being cold when it was actually warm (Ryan and Revilla 2003). The fact that a certain percentage of each anthocyanin form is found in grape skins of a particular cultivar suggests genetic basis for anthocyanin composition within the berry that is consistant year to year (Pomar *et al.,* 2005). Support for the genetic-based accumulation of particular anthocyanin forms in particular abundance of each form was found in a study of a large collection of *Vitis* germplasm which separated species and species' hybrids on the major anthocyanin form accumulated (Liang *et al.,* 2008). However, Ryan and Revilla (2003) suggested that percentages of Petunidin-3-O-glucoside and Delphinidin-3-O-glucoside could be used in predicting whether the growing season in question had been warm or cold due to an observed fluctuation of percentages of these two monoglucosides in Cabernet Sauvignon and Tempranillo cultivars when temperatures were different from one season to the next. Interestingly, both of these monoglucosides appear on the same branch of the anthocyanin biosynthetic pathway (Boss *et al.,* 1996a).

Total anthocyanin accumulation is known to vary year to year, or be vintage-dependent. A significant difference in amount of anthocyanin accumulated was observed from one year to the next and could not be explained by growing site which meant that it was independent of the sites where the grapes were grown for the study (Brossaud *et al.*, 1999).

2.3.2 Measurement of anthocyanins

Anthocyanins are most pronounced in their coloration, in other words have the maximum absorbance at a 520 nm wavelength when they are in solution at pH 1.0 (Wrolstad, 1976). This is why acidic solutions are generally utilized to help measure as high a percentage of the total anthocyanins in the berry extract as possible. If a solution were to be at pH 4.5, the anthocyanin has lost a proton and would appear colorless just like flavonols would in solution (Boulton, 2001). The use of an acidic solution for extracting anthocyanins is still practiced to this date (Mazza *et al.*, 1999; Fukumoto and Mazza, 2000). However, there is controversy as to whether acid based extraction should be used due to reported hydrolysis of anthocyanins in acid solution or feeling that anthocyanins arose from flavonols or proanthocyanidins contained in the same acidic extract can occur when using acid-based extraction techniques (Revilla *et al.*, 1998).

An explanation for the great variability in wine color especially at the moment of bottling is due to the fact that each form of anthocyanin possesses its own unique spectral properties and this allows for the grapes and wine to take on a large range of coloration. Quantifying amount of a particular anthocyanin present in grapes or wine is impossible by chromatography alone, but when combined with the use of spectrophotometry then quantification of a particular anthocyanin is possible. Analysis of the different anthocyanin conformations in grapes can allow for selection of grape cultivars that provide good coloration when made into wine (Hebrero *et al.*, 1989).

In addition to the spectrophotometer utilizing-methods, individual anthocyanins can be detected using the HPLC. Measurements of the anthocyanins were conducted on red wine samples injected directly into the HPLC. Individual anthocyanins can be recorded by HPLC. Detection was carried out at 313 and 546 nm using a UV-visible light

detector. A 25-minute linear elution gradient was used starting at 11% methanol concentration in water:formic acid (90:10) and ending at 36% methanol in water:formic acid (90:10) in the study of Gonzalez-San Jose *et al.* (1990).

In another anthocyanin analysis on the HPLC, samples were prepared by using a solution containing primarily ethanol with an acid pH of 3.2. The HPLC analysis of anthocyanins used two different buffers to elute individual anthocyanins. Solvent A consisted of formic acid and water (10:90), while Solvent B consisted of formic acid, methanol, water (10:50:40). Solvent A began at 72% of the gradient composition to 1% of the gradient composition in a 50 minute time period (Guidoni *et al.*, 2002).

In an analysis in which both flavanols and anthocyanins were detected, an HPLC-DAD was used in peak analysis and HPLC-MS was used in mass detection. For flavanols, monomers were identified in the ether fraction while flavan-3-ol derivatives were extracted with ethyl acetate. For flavanol and their derivatives analysis, Solvent A consisted of 4.5% formic acid in water and Solvent B consisted of Solvent A in acetonitrile (9:1). Solvent B was 0 to 50% of the gradient compostion for 40 minutes, 50 to 100% for 50 minutes and isocratic for another 20 minutes. For the anthocyanin analysis, the gradient consisted of: Solvent A) water:formic acid (9:1), and 35-95% over 20 minutes of run-time of Solvent B) methanol/water/formic acid (45:45:10) (Perez-Magarino and Gonzalez-San Jose, 2004).

2.4 Organic Acids in Grapes and Wine

2.4.1 Introduction to organic acids in grapes and wine

The most prevalent organic acids in grapes are tartaric and malic acids. It is worth noting that tartaric acid accumulation is restricted to a small number of plant species and tartaric acid certainly serves a vital role in wine (DeBolt *et al.*, 2006). Other noteworthy organic acids are ascorbic acid, oxalic acid, and citric acid. Tartaric acid and malic acid have been studied in great detail to determine the nature of their biosynthesis and accumulation in plants such as grapes.

Regarding tartaric acid, it was first established that a small amount of ^{14}C-label from L-ascorbic acid was observed in (+)-tartaric acid and so it was established that there was indeed a biosynthetic pathway leading from L-ascorbic acid to (+)-tartaric acid (Loewus and Stafford 1958). Later experimentation of a similar nature revealed 72 percent of the radioactivity associated with L-ascorbic acid-1-^{14}C was present in the resulting tartaric acid. The results suggested that L-ascorbic acid could be efficiently converted to tartaric acid. Saito and Kasai (1969) also found that radioactivity was contained on the carboxyl groups of tartaric acid which would corresponds to the 1st through 4th carbons, C-1 to C-4, of L-ascorbic acid and suggested that this part of L-ascorbic acid is directly converted to tartaric acid. While experimentation has feed radioactive ascorbic acid to grape bunches, Hale (1962) discovered that grapes were capable of tartaric acid biosynthesis independent from the grapevine. Review of research on the biosynthesis of tartaric acid has revealed a cleavage at the C-4 and C-5 of L-ascorbic acid by hydrolysis for formation of L-tartaric acid (Loewus, 1999). Conversion of both dehydroascorbic acid and L-ascorbic acid to tartaric acid was found to be similar in nature (Saito and Kasai, 1984).

There is similarity between the synthesis of tartaric acid and the synthesis of oxalic acid. Oxalic acid is known to also trace its biosynthesis from L-ascorbic acid. However, its cleavage is at the C-2 and C-3 by oxygenase and hydrolyase activity to utilize the C-1 and C-2 of L-ascorbic acid (Loewus, 1999).

Tartaric acid has properties which make it unique when compared to noteable organic acids found in grapes, one of those being malic acid, in particular. In comparison to malic acid which is typically second in quantity in the grape at harvest, tartaric acid is inert to microbial metabolism unlike malic acid. Tartaric acid is also not oxidized like malic acid is during fermentations which allows tartaric acid to serve the important role of keeping wine pH low either at its level in the berry at harvest or through addition of more tartaric acid during winemaking (Banhegyi and Loewus, 2004). The three acids, tartaric, malic, and citric, which are all synthesized in grapes are all carried through fermentation, but it is only tartaric acid which is inert to the entire process with both malic and citric acids being metabolized and oxidized during fermentation. Aged wine

contains approximately two-thirds the amount of tartaric acid as the grapes it was made from due to tartrate precipitation (Conde *et al.*, 2007).

Compared to tartaric acid which remains at a stable level in grapes post-veraison, the levels of malic acid vary greatly as grapes mature (Conde *et al.,* 2007). The biosynthetic formation of malic acid takes place in an entirely different fashion than that of tartaric acid biosynthesis known to begin with ascorbic acid. No radioactivity was found to be associated with malic acid when ^{14}C-labeled L-ascorbic acid was introduced to a berry cluster and while the majority was found in tartrate and the second most percentage was in oxalate, absolutely no radioactivity was found with malic acid (DeBolt *et al.*, 2004).

Malic acid is known to accumulate in the vacuole being that is used by the plant in processes such as photosynthesis. As vacuole size increases coincide with cell expansion, the amount of malate that is able to accumulate also increases. Malic acid was found to accumulate in young berries and reach its maximum level in berries at veraison followed by a decline post-veraison (Melino *et al.,* 2009). As reviewed in Conde *et al.* (2007), the levels of malic acid in grapes have been noted as being higher in cool regions; and for this reason, the grapes in warm regions are lower in their acidity. Enzyme studies reveal that the maximum regulated levels of malic acid in grapes takes place at temperatures between 20°C and 25°C. High temperatures lead to inactivation of enzymes regulating malic acid accumulation in grapes; it was found that a temperature of 45°C inactivated malic enzyme as well as PEP carboxylase which suggested that accumulation of malic acid would happen in cooler conditions (Lakso and Kliwer, 1975).

Oxalic acid was found to increase in the early stages of berry development but decline some in later stages of berry ripening. Melino *et al.* (2009) concluded that due to the fact that there is a great distribution of oxalic acid in tissues of the grape vine other than the grape berry suggests that the grape berry has a less significant role in calcium storage than other plant organs. Further evidence for the minor role of calcium sequestration in grapes berries is found by the localization of calcium in roots of grapevines that serve the purpose as a temporary sink capable of regulating calcium availability in the plant (Storey *et al.,* 2003).

2.4.2 Intermediates of the tartaric acid biosynthesis from L-ascorbic acid

In biosynthesis of tartaric acid starting with L-ascorbic acid, there are intermediates which research has shown to proceed conversion of L-ascorbic acid to tartaric acid. One such intermediate is 5-keto-D-gluconic acid, known as 5-keto-IA. The research established 5-ket0-IA as a metabolic product of L-ascorbic acid in slices of grapes (Saito and Kasai, 1984). In the study of Saito and Kasai (1984), it was observed that 5-keto-IA behaved metabolically like L-ascorbic acid with oxidative cleavage of the compound to form tartaric acid. Another intermediate in ascorbic acid metabolism to tartaric acid, L-idonate, was found to convert to 5-keto-D-gluconic acid by L-idonate-dehydrogenase (L-idnDH) in a study which sought to elucidate enzymes governing the conversion of ascorbic acid to tartaric acid in *Vitis vinifera* (DeBolt *et al.,* 2006).

2.4.3 Observation of oxalic acid within the plant

The process by which oxalic acid associates with calcium is considered a biomineralization process. It involves specialized cells which are involved with crystal formation. The specialized cells and the molecules they contain are referred to as the organic matrix (Webb, 1999).

Raphide bundles originating from leaves of *Vitis labrusca* and *Vitis mustangensis* Buckley were identified via X-ray powder defraction as calcium oxalate monohydrate. Determination of this particular molecular arrangement was based on molecular analysis of the bundle center where calcium was the prevalent cation. Calcium was also found to be contained in the surrounding matrix further lending support to the role of calcium oxalate monohydrate as means of calcium storage, and potassium was only found at the ends of the crystals which would rule out its proposed role as the primary cation involved in formation of crystals with oxalate (Webb *et al.*, 1995). The calcium levels in the vacuole are on the order of a thousand times higher than in the cytoplasm and this compares to potassium levels only on the order of ten times higher in the vacuole which gives weight to calcium salts formation in lieu of potassium salts formation (Storey *et al.*, 2003).

Idioblasts are cells having their vacuoles organized in a manner which promotes the formation of crystal chambers. Chambers which promote nucleation of calcium oxalate are found inside membrane compartments and are called crystal chambers. The chambers contact the crystals and the chambers housing the crystals are within the vacuole of cells producing crystals. Demineralization studies have found evidence in support of crystal formation due to crystal chambers being unique in their structure (Webb *et al.*, 1995). In grape berries, there is evidence that crystal formation is a directed process and that formation of calcium oxalate in crystals other than raphide and druse does not happen (DeBolt *et al.*, 2004).

An explanation for such an ordered production may lie in the fact that it is likely that biosynthesis of oxalic acid which begins metabolically with ascorbic acid has to be a directed process within the grape berry. There are multiple cell types and it is probable that idioblasts would need to interact with surrounding cells of the mesocarp in order to obtain the ascorbic acid to be metabolized to form oxalic acid (DeBolt *et al.*, 2004). DeBolt *et al.* 2004 concluded that conversion of ascorbic acid to oxalic acid only occurred in specialized cell types in the grape and that oxalic acid synthesis is only limited to calcium oxalate crystal formation; it was also noted that tartaric acid synthesis was throughout the mesocarp, suggesting a less specialized role in tartaric acid synthesis.

It was established in Webb *et al.* (1995) that the crystals of calcium oxalate monohydrate in grape leaves were raphide crystals. Arnott and Webb (2000) used this study as a basis for their investigation into specific crystal morphology involved with calcium oxalate crystals. Under the microscope, it was observed that when turned along their central axis which is the long axis, the appearance of a raphide was seen during rotation which was the evidence Arnott and Webb (2000) used for calcium oxalate crystals exhibiting raphide twinning in grapes.

2.4.4 Measurement of organic acids

It is necessary to measure organic acids in a manner which takes into account there are salts for some of the organic acids. Analysis of Tartaric and Malic acids involved using 1 M HCL to extract their salt forms once free salt forms had been extracted using 80%

ethanol. Pulp and skin was analysed for contents of these two organic acids (Iland and Coombe, 1988).

In particular, it is important to dissolve the salts of oxalic acid so that free oxalic acid and its salts can be measured (DeBolt *et al.,* 2004). In Melino *et al.* (2009), liquid nitrogen was used to powderize frozen berries. The extracted used 3% weight by volume MPA to 1 mM EDTA to a final volume of 10 mL. After centrifugation and filtration, 2 mL of total volume remained in the original tube. The pH of the berry extracts was adjusted with 8.7 M ortho-phosphoric acid. To dissolve oxalic acid crystals, samples were mixed for 2 hours. For HPLC analysis of the organic acids, a gradient of two solvents was used. Eluent A consisted of 25 mM KH_3PO_4 with 0.1 mM EDTA at pH 2.5, and eluent B contained 100% methanol. Eluent A was decreased by 10% while eluent B composed 10% of the gradient, and this was reversed so the final content was eluent A at 100% and eluent B at 0% (Melino *et al.,* 2009).

It is important that acid extraction is done in order to dissolve any particular salts, and acid extraction was not done in studies such as Saito and Loewus (1989) and this potentially implies that oxalate salts would not have been detected due to only measuring free organic acids. Acid extraction can be done by methods such as the method of DeBolt *et al.* (2004) which used 0.5 M phosphoric acid to dissolve oxalate crystals with two hours of time on a rotating mixer to effectively dissolve the oxalate crystals.

2.4.5 Processing effects on organic acids

Done in a timely manner, the loss of organic acids through ongoing enzymatic processes can be halted. L-ascorbic acid in particular is at risk of losses and is even used as a marker of the state of post-harvest produce. In fruits the lower pH of the berry is known to create a more stable environment for the organic acids. The sooner fruits are put into cold storage if intended for sale as simply produce; the lower the post-harvest loss of organic acids. The same is true for canned fruits; sterilization will halt enzymatic activity allowing for months of stability of the product and freezing prevents any more losses of organic acids that might be incurred (Davey *et al.,* 2000).

When examined for quantification purposes, or measurement of organic acids, it was found that there was not a significant difference between fresh and frozen samples in their respective levels of oxalic, tartaric, malic, and ascorbic acid (Melino *et al.*, 2009).

2.4.6 Bacterial fermentation of organic acids

To discourage microbial spoilage and if wine has high acidity level due to being made from grapes grown in a cool region, malolactic fermentation is used reduce the levels of malic acid in the wine (Conde *et al.,* 2007). Malolactic fermentation is when bacteria convert malic acid to lactic acid. Commercial strains of wine yeast, *Saccharomyces cerevisiae*, can convert between 0 and 40% of the malic acid supply during fermentation. Due to significant advances in genetic engineering, there is the possibility to apply alcoholic and malolactic fermentations at the same time in the winemaking process. This is allowed in the genetically modified yeast by having an enzyme inside of the yeast cell that can convert malic acid to lactic acid. In fact, the genetically enhanced yeast, ML01, converted malic acid to lactic acid with only residual levels of malic acid remaining in the wine in only 60 hours compared to the malolactic bacteria Lalvin 31 taking 21 days to complete malolactic fermentation (Main *et al.,* 2007).

Citric acid can be converted to other compounds such as acetic acid by wine microorganisms. Bacteria that ferment citric acid to acetic acid generally produce much of the vinegar in wine. The process of citric acid converting to acetic acid is not desirable in wine because of the undesirable taste of excessive amounts of acetic acid in wine. Other causes of excessive acetic acid levels in wine include lactic bacteria producing acetic acid from residual sugars in stuck fermentations and vinegar bacteria producing large amounts of acetic acid from ethanol in cases where there is too much exposure to air (Conde *et al.,* 2007).

2.4.7 Timing of malolactic fermentation during winemaking

Addition of malolactic bacteria at the end of fermentation is recommended due to malate consumption by the yeast (Main *et al.,* 2007). Another consideration is the opinion of those tasting wines; Californian winemakers have claimed that the wine's sensory

attributes suffer if bacterial malolactic fermentation does not follow the yeast alcoholic fermentation (Kunkee, 1991). How much time after alcoholic fermentation began is questionable; the preferred time of inoculation of must with malolactic bacteria is the second or third day after alcoholic fermentation began when there are the low ethanol, low SO_2 and warm fermentation conditions which favor malolactic fermentation (Dharmadhikari, 1992).

2.5 Treatments Intended to Increase Berry Phenolics and Improve Berry Coloration

2.5.1 Treatments used in previous research

Four such treatments for increasing berry phenolics and improving berry coloration have included use of abscisic acid (ABA), benzothiadiazole (BTH), ethanol, and ethephon. Applications to the grapevine are made at veraison and post-veraison at times preceding harvest. In some cases the treatments show direct correlation with the active compound and berry phenolic level and berry color. The four different compounds used in treatments to improve berry quality will now be expanded upon.

2.5.2 Abscisic acid treatment

It was found that the levels of abscisic acid (ABA) were found to correlate with anthocyanin accumulation in the grape berry (Pirie and Mullins, 1976). Therefore, it makes sense that an exogenous application to the plant could indeed allow for increased anthocyanin biosynthesis. The study of Ban *et al.* (2003) of ABA application to grapes at veraison showed increase in expression in several enzyme genes in the berries' skins, including UDP glucose-flavonoid 3-0-glucosyl transferase (UFGT), known to be critical for anthocyanin biosynthesis (see Boss *et al.*, 1996a,b); and, indeed, there was increased anthocyanin accumulation in berries that received ABA treatment. Further support for an increase in anthocyanin biosynthesis enzymes genes activity was found in Jeong *et al.* (2004), which showed increased expression of VvmybA1, a gene known to be directly coordinated with the expression of other anthocyanin biosynthesis enzymes genes and also anthocyanin accumulation.

Anthocyanin accumulation is often compared to sugar content of grape berries due to accumulations of both when the berry behaves as a sink at veraison. This is explained in Downey *et al.* 2003a, which describes the nature of the berry from veraison onwards as a dispersal strategy for the plant in which anthocyanin accumulation and increased sugar content aid in the berry's dispersal. It is for this reason that ABA, which is correlated to anthocyanin accumulation, also requires a supply of sugars in order for phenolic content and anthocyanin content to increase in the plant. The synergy of ABA and sucrose to enhance anthocyanin accumulation was demonstrated in leaf sections in the study by Pirie and Mullins (1976). This could very well be the reason why it is essential that ABA application takes place at veraison when the grape berry has become a sink organ of the plant.

ABA application might be most practical in terms of noticed increase in grape coloration when applied in a hot climate that is not conducive to anthocyanin accumulation. Evidence for this theory has been found in studies which showed restored levels of anthocyanin accumulation in heat-treated clusters. In the study by Yamane *et al.* (2006), it was observed that levels of ABA were a significant amount higher, approximately by 1.6 times, in grapes at 20°C versus the grapes at 30°C. Temperature linkage with ABA suggests the possibility that lower temperatures favor ABA biosynthesis and/or prevent its degradation.

2.5.3 Benzothiadiazole treatment

The compound benzothiadiazole (BTH) serves as a functional analog of salicylic acid (SA) to induce a systemic immunity in the plant (Kunz *et al.*, 1997). There are several plant species which show systemic acquired resistance (SAR) when treated with BTH against a spectrum of plant diseases including wheat, arabidopsis, tobacco, and bean plants. The affect that BTH produces in the plant is formation of pathogenesis related (PR) proteins because it is an analog of SA and additionally enzymes of secondary metabolic pathways such as phenylalanine ammonia-lyase (PAL) which is involved early in flavonoid biosynthesis (Iriti *et al.*, 2004). Plant secondary metabolites have been linked to traits of plant defense. Phenylpropanoids form a major class of secondary

metabolites in plants. The enzyme phenylalanine ammonia-lyase is the first enzyme involved in the phenylpropanoid pathway (Gozzo, 2003).

One phenolic class shown to increase with treatment of BTH is anthocyanins which are one of the secondary metabolites produced by plants. In fact, the whole anthocyanin content was found to increase with BTH application at the end of veraison with the prevalent form of malvidin glucosides increasing more than 100% compared to the control plants (Iriti *et al.*, 2004).

The mode of delivery of an exogenous treatment directly onto berries was done using ethephon at veraison. It was found to stimulate genes that are related to anthocyanin biosynthesis (El-Kereamy *et al.*, 2003). A similar sort of process has been found to take place with BTH treatment on bean plants, which suggests that there is stimulation of the ethephon (ethylene) transduction pathway with BTH treatment (Iriti and Faoro 2003).

2.5.4 Ethanol treatment

Spraying ethanol at veraison has resulted in increases in ethylene production by the plant (Chervin *et al.*, 2001). Likewise, the use of ethanol with the ethylene precursor ethephon has enhanced the overall affectiveness of ethephon treatment through better diffusion of ethephon across the grape cuticle (Farag *et al.*, 1992).

Treatments using ethanol are done at veraison where there is approximately 50% coloration (El-Kereamy *et al.*, 2003). Water having a content of 5% ethanol for use as a treatment produced the result of a rapid increase in the ethylene content of grapes. Also, grapes clusters sprayed with 5% ethanol contain greater levels of red pigmentation in their skins than the control clusters (Chervin *et al.*, 2001). In their study, Chervin *et al.* (2001) also observed greater color intensity in wines made from 5% ethanol-treated grapes than wines made from control grapes. The effectiveness of 2.5 to 10% ethanol treatment was tested in a three year study. It was found that 5% ethanol treatment produced the greatest wine acidity, darkest grape coloration, and darkest wine coloration of all treatments. Furthermore, wine made from the 5% ethanol treated grapes over three concurrent vintages was significantly darker than wine made from control grapes of the

respective vintages, for a look at the improvement brought on by the ethanol treatments, see Figure 2.1 (Chervin *et al.*, 2004).

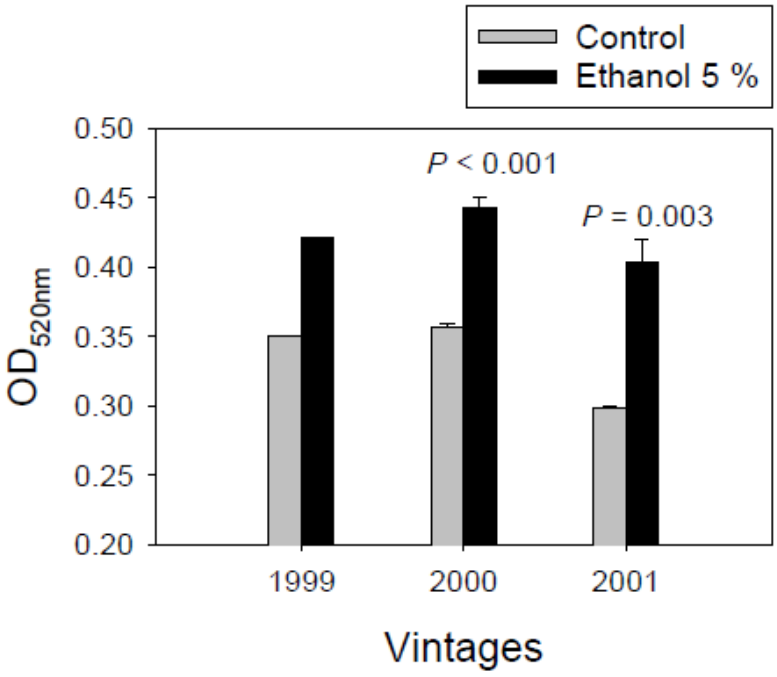


Fig. 5. Effect of spraying ethanol after mid-veraison on the wine OD at 520 nm. Measures were performed in 1 mm quartz cuvette without dilution, n = 3, except for 1999 where n = 1, error bars represent SE, and *P* is the probability that the control mean is equal to the treatment mean.

Figure 2.1. Graph of three vintages of an ethanol spray study by Chervin *et al.* (2004).

Blot analysis revealed greater expression of UFGT in ethanol treated plants; UFGT is a critical enzyme of anthocyanin biosynthesis. UFGT was expressed to a higher degree in ethanol treated plants than control plants for a period spanning from the first day of treatment until twenty days later. There was no observed effect on other enzyme genes of anthocyanin biosynthesis that occur earlier in the anthocyanin biosynthesis pathway (El-Kereamy *et al.*, 2003).

Regarding the environment in which ethanol application may be most ideal; it was observed that when the weather was cloudy and cooler in late summer that the affect of ethanol treatment on berry coloration was greater than in conditions where it was sunny and warm. Therefore, the potential usage of ethanol to improve coloration may be highest in areas having cool climates where the likelihood of cloudiness and coolness at the end of the summer would be greatest (Chervin *et al.*, 2001). As a note, this observed climatic influence on treatment affectiveness appears to be opposite of the influence found with ABA. In ABA treatments, levels of anthocyanins were restored to what they typically were in most years in the circumstance of being heat-stressed. The heat-stressed grapes were no longer producing anthocyanin, suggesting that either ABA biosynthesis is favorable at cool temperature conditions or that degradation of ABA advances faster in hotter weather (Yamane at *al.*, 2006).

When ethanol is used in conjunction with ethephon, it was found that coloration was improved most in the season which was warmest. This suggests that although ethanol may be more affective in cool conditions, its ability to deliver the ethylene precursor into the berry can allow for higher ethylene levels thereby bringing about signaling needed to stimulate anthocyanin biosynthesis in berries (Farag *et al.,* 1992).

2.5.5 Ethephon treatment

Ethephon treatment has been most associated with accelerating ripening in fruits. In grape berries treated with ethephon eight weeks after full-flowering and possessing coloration, there was a much higher sugar to acid ratio which signifies that fruit is riper. It was also found that the ripening effect attributed to ethephon was most pronounced in cultivars whose slow growth phase corresponding to the time preceeding and during verasion was extended versus brief (Hale *et al.,* 1970). To conclude their findings, Hale *et al.* (1970) found that berries which had coloration at time of application during veraison were riper than control berries while berries that were still green at the time of application at veraison were less ripe in comparison to control berries.

Coloration of grapes and wine made using the grapes is known to improve with ethephon treatment of the grapes. Anthocyanin content was found to be greater in the ethephon

treatment samples than control samples (Weaver and Montgomery, 1974). When applying ethephon with different water regimes, it was found that ethephon increased grapes' anthocyanin content over control grapes in every irrigation treatment and in both seasons (Hardie *et al.*, 1981). Wines made from the ethephon treatment grapes had darker color than wine made from the control grapes and this might be due to the affect that ethephon might have on increased production of the monomeric anthocyanins malvidin-3-glucoside and peonidin-3-glucoside which are considered to be stable anthocyanins (Powers *et al.*, 1980).

There are other effects that have been noted with ethephon application. Total acids (TA) of ethephon treated samples decreased in comparison to the control samples (Weaver and Montgomery, 1974). In one of two growing seasons, ethephon accelerated soluble solids accumulation in grape indicating hastened ripening. Ethephon also decreased TA in each water regime in both seasons (Hardie *et al.*, 1981). Treatments of ethephon decreased TA levels a month after veraison. The ethephon treatments also increased ripening as indicated by the total soluble solids content (Shulman *et al.*, 1985). In Hale *et al.* (1970), the results showed an increase in the sugar content to berry acidity ratio.

Berry firmness is one measurement that has been done in gauging quality attributes of grapes treated with ethephon because of the ripening effects associated with ethephon. It was observed in Tokay variety grapes that all levels of ethephon used in treatments resulted in grapes that were less firm than the no ethephon control. In the variety Emperor, all treatments except for the low level 100 ppm ethephon in one of the three vineyards where the Emperor variety was located (Jensen *et al.*, 1975). In a study involving Crimson Seedless table grapes, two clones of this variety showed a reduction in their firmness with ethephon treatment. One of the Crimson Seedless clones showed no change in its firmness when treated with ethephon (Jayasena and Cameron, 2009).

There was an observation of affectiveness of ethephon treatment based on the location. This was found in the study of Jayasena and Cameron (2009) where the controls at one site had better coloration, but with ethephon application the grapes at both sites had comparable color.

2.6 Conclusions

Although a great amount of research of phenolic compounds and organic acids in wines and wine grapes has been done due to it being a crop of economic importance worldwide , there are still many fundamental processes that need to be established and implemented in order to produce quality wine on a cultivar basis. One good example is the lack of a standardized system of measuring the tannins of wine and wine grapes (Downey *et al.,* 2006). The methods currently used vary in the tannin concentrations reported for each wine when the methods are run simultaneously to compare their results (Kennedy *et al.,* 2006). Standardized systems for measuring phenolics can allow for industry-wide reporting of obtained values from wines and allow recommendations of appropriate ranges of phenolic groups in wines. Another benefit of standardizing such a system would be to allow for estimation of when wine will be at its peak quality. The ratio of tannins and anthocyanins is known to influence the tannin quality, mellowness and balance (Cheynier *et al.,* 1998). Another effect of phenolic composition on aging wine is determined by stability of coloration which is based on polymeric pigment formation (Somers, 1971; Timberlake and Bridle, 1976). Having a better idea of when a wine should be available to the consumer and when it will be at its peak quality are both connected with wine phenolic content and are important for success in the wine market.

Anthocyanins are important as they provide the coloration associated with wine varietals and are the one of the molecules contained in polymeric pigments, the other molecule being a flavonol or condensed tannin composed of many flavonol subunits (Harbertson *et al.,* 2003). Anthocyanin composition of grapes at harvest can also suggest weather conditions during berry ripening and the effect that this may have on wine coloration is yet to be determined (Ryan and Revilla, 2003). However, it has been determined by analysis of *Vitis* germplasm that genetic background has a vital role in the expression of anthocyanins in the grape berry (Liang *et al.,* 2008). The fact that the genetics of the grape berry have such a profound influence on anthocyanin content of the berry is a reason why fingerprinting cultivars for phenolic compounds such as anthocyanins is possible. Fingerprinting has worked in models looking at prediction of cultivar based on

their anthocyanin composition to the extent that the models predicted the correct cultivar in all samples analyzed (Arozarena *et al.*, 2002; Ryan and Revilla, 2003).

Organic acids hold great importance in the winemaking process, providing the acidity needed to prevent spoilage by microbes. Tartaric acid is the most predominant organic acid in the grape berry at harvest and holds the distinction of being inert to microbial metabolism while undergoing the fermentation process to make wine (Banhegyi and Loewus, 2004). Being inert to metabolism by microorganisms that are naturally present in the environment, tartaric acid can be added to wine if the wine pH is too high and needs to be lowered. Malic acid is the second most abundant organic acid at harvest, but unlike tartaric acid, malic acid is readily metabolized by wine microorganisms. To prevent microbial spoilage and reduce high acidity found in winegrapes grown in cooler regions which have accumulated higher malic acid content, malolactic fermentation has been considered essential (Conde *et al.,* 2007). Currently, the advancements in genetic modification of organisms has lead to the development of wine yeasts that have been genetically modified to metabolize malic acid due to possession of an enzyme capable of converting malic acid to lactic acid which is the same manner bacteria carrying out malolactic fermentation by converting malic acid to lactic acid (Main *et al.,* 2007). Being able to reduce organic acids which can be metabolized undesirably by microorganisms and spoil the wine can allow for more successful winemaking by helping to eliminate known factors contributing to wine spoilage.

Treatments to improve berry coloration and increase berry phenolic content to sustain a higher quality wine can include use of plant hormones such as ABA especially in circumstances where optimal conditions for anthocyanin accumulation are lacking such as in hot temperature climates where ABA treatment has restored anthocyanins to levels normally found in the grapes (Yamane *et al.,* 2006). Treatments including ethanol and ethephon which cause ethylene evolution within the grapevine are promising for widespread application in the future being that both compounds are readily available and inexpensive. Using ethanol in the vineyard setting at a 5% concentration saw consistent improvement in the coloration of grape berries treated with ethanol. The more newly researched compounds, benzothiadiazole, is considered to be an inducer of systemic

acquired resistance. Systemic acquired resistance creates an increase in secondary metabolite levels; one such group is the flavonoids which include anthocyanins. Anthocyanins were found to increase as a result of application of benzothiadiazole (Iriti *et al.,* 2004). The ongoing use of treatments to improve coloration and increasing overall phenolics in berries is necessary for consistency in wine quality from season to season. While the practice of treatments to improve compounds like phenolics in grapes may not be commonplace, applications of the aforementioned compounds can make a difference in usefulness of grapes for winemaking. In unfavorable weather conditions which stress grapevines such as high temperatures or water deficit, the use applications which have been shown to increase phenolic content of berries including improvement of coloration through increase in berry anthocyanins can allow for the grape harvest of that season to remain sound for the purpose of wine production.

CHAPTER 3: WINE PHENOLICS

3.1 Introduction

It is well-known that evaluation of wine quality is based on an array of sensorial attributes. Arguably, the most important are coloration of the wine and degree of astringency and bitterness of the wine. Coloration is associated with particular wine varietals and wine types. Astringency and bitterness are likewise associated with type of wine and additionally wine vintage. The overall impact that wine phenolics has on these important attributes is well-documented.

Studies examining coloration of wines associate deep redness to new wines (Jackson, 2008). This is due to the particular anthocyanin content and anthocyanin co-pigmentation that occurs in new wines (Boulton, 2001).

When the astringency and bitterness of wines have been examined, there has been a great deal of correlation with amount of tannin in the wine (Landon *et al.,* 2008). Astringency is most related to the tannin content of the wine. Bitterness has been linked to several wine compounds with the most predominant and significant to taste being total phenol content. The flavan-3-ols, (+)-catechin and (-)-epicatechin, are wine phenolics that are considered to impart a bitter taste (Thorngate, 1997).

The phenolic groups of polymeric pigments are important in aging potential of wines. Monomeric flavonoids and oligomeric proanthocyanidins which are respectively flavan-3-ols and oligomers composed of flavan-3-ol subunits, in association with anthocyanins, compose small polymeric pigments. Polymeric flavonoids having subunits of flavan-3-ols, usually referred to as condensed tannins, in association with anthocyanins are what make large polymeric pigments in wine. Their presence is notable in providing the stable coloration familiar in wines that age well. However, variabilty in the relative ratio of small polymeric pigments and large polymeric pigments does exist within the same variety of grape used for wine and the overall importance of the relative abundance of each in wine is still being determined (Harbertson *et al.,* 2003).

It is the overall level of wine phenolics which include the notable groups of anthocyanin, tannin, small polymeric pigments and large polymeric pigments which often define a particular wine type such as white wine or red wine. Often the level of astringency can be correlated as white, red, or full-red wine due to tiers that exist for each in the amount of tannin that the wines contain (Landon *et al.,* 2008).

The ability to group wines into broad subcategories is why determination of total wine phenolics has been done in the past. However, it is of importance to note that less variation in total phenolics exists between particular cultivars of a given wine type, such as red wine. In fact, the differences of phenolics between cultivar whether one cultivar has a greater abundance or less abundance of phenolics than another was found to be different from vintage to vintage suggesting that a wine cannot be specifically linked to the wine grape cultivar (Mazza *et al.,* 1999). While there is significance in total phenolics in taste attributes, and a wine of a cultivar should have a total phenolics value that is reflective of that cultivar and wine type; the ideal analysis of wine phenolic composition should account for multiple groups of wine phenolics.

The use of methods of analysis which are practical in number of potential samples that can be tested during one assay to quantify the levels of phenolics has been done in more recent research involving phenolics. A number of methods utilize the light absorbance properties of phenolic compounds to quantify content of a phenolic group within a given sample and the light absorbance is measured using a spectrophotometer (Mazza *et al.,* 1999; Harbertson *et al.,* 2003; Landon *et al.,* 2008).

The analysis of wine phenolic groups utilizes techniques that discern the concentration of the particular group in the sample. The assay developed by Hagerman and Butler (1978) used bovine serum albumin (BSA) protein to measure tannin levels because tannin precipitates protein. This assay is still utilized as a way of measuring the tannins in wine an example is the use of the Hagerman and Butler (1978) method as part of the analysis involved in determination of the polymeric pigment content of wines in the study by Harbertson *et al.* (2003).

3.2 Materials and Methods

3.2.1 Wines used in the analysis

The red wines used in this analysis were from Washington, California, and mid-West. The majority of wines were from the state of Kentucky. Wines from the state of Kentucky were deliberately selected because of the interest in examination of wine phenolics of grapes from this region. In order to have a representative sample size for each varietal, it was decided that the wines be classified solely by varietal and not by the growing region or state. The red wines ranged in age from young at only six months maturation to more matured reds that were from the vintage of 2004 which would have made the oldest wines five years old at the time that the wine tastings on the campus of the University of Kentucky in Lexington, Kentucky.

Wines made from grapes obtained from one varietal were investigated and the five varietals examined were Cabernet Franc (n=10), Cabernet Sauvignon (n=28), Chambourcin (n=14), Merlot (n=8), and Norton (n=16). Preference of usage of particular wines in analysis was given to the varietals: Cabernet Franc, Cabernet Sauvignon, Chambourcin, and Norton. The aforementioned are the four red wine grape cultivars with the most acreages per cultivar in the state of Kentucky; and for this reason, they are reflective of the most commonly produced red wines by cultivar in our state (Smigell *et al.*, 2008). Merlot was utilized because it is a common red wine worldwide and it shares similar taste attributes as the other varietals of wine used in this study. It proved useful as a wine to use during wine tastings which had wines examined in the phenolics analysis of this study. In the tastings, it was included along with the wines of different grape cultivars.

3.2.2 Sampling of wines

While no official protocol for taking wine samples for use in phenolic analysis exists, certain measures were taken to make results as valid as possible. The samples were all obtained from full wine bottles that had just been opened and this was necessary for good analysis. Each sample was placed in three 1.5 mL eppendorf tubes so that each tube was

full. The primary reason for use of full wine bottles and completely filling centrifuge tubes with the wine samples is that the process of oxidation of wine can be fast for wine of any age. In particular, the noticeable sign of discoloration of wine to a brown, orange, or yellowed tinge is something that is both non-aesthetic and indicative of the oxidation of wine phenolics. Wine anthocyanins are known antioxidants and the presence of oxidative radicals like hydrogen peroxide can cause for anthocyanins to become oxidized. In fact, anthocyanins that have been oxidized cannot be measured at the wavelength used for quantification (Van Acker *et al.*, 1996). Due to oxidation anthocyanins are unable to be measured and a noteable change of coloration in oxidized wine suggests that the anthocyanins are no longer providing the coloration that they did prior to being oxidized.

Another rational to obtain samples from full wine bottles for usage in phenolics analysis is the fact that sedimentation is increasingly more common with samples obtained further from the top of the bottle. Not all wines are filtered and sedimentation is also a natural process occurring over time. Filtering to eliminate sediment was not done in the analysis because of the assumption that sediment found in the wine would include some wine phenolics and upon removal would make the sample of that wine invalid. However, it was felt that samples should be homogenized before use in phenolics analysis because large, macromolecular sediment particles could interfere with generating an appropriate spectrophotomeric reading.

Another of the considerations applied to samples for use in the wine phenolics analysis was the storage of the samples. To minimize oxidation and microbial processes which could affect results of the wine phenolics analysis, the samples were kept in a 4°C refrigerator until being needed in the assay. Phenolics analysis was done as soon as possible after the wine samples were prepared. Phenolics analysis of samples was done in a timely manner to avoid disagreeable processes that could happen to the wine and to reflect the composition of the wines which were judged by tasting panel later in the same day the samples had been placed in the 1.5 mL centrifuge tubes. Analysis of the samples was performed in a lab in the Agricultural Science Center North building at the University of Kentucky.

3.2.3 Materials

Multiple wineries from the state of Kentucky made contributions to the analysis by their donation of bottles of wine. The majority of the wines came from Kentucky and the surrounding states and a respectable minority of the wines were from California, Washington, and Oregon. Wines originating from out of the state of Kentucky were purchased at local wine retailers. Reagent grade sodium dodecyl sulphate (SDS), HCl, ferric chloride hexahydrate, ethanol, glacial acetic acid, NaOH, bovine serum albumin (BSA) were purchased from Fisher Scientific, Fair Lawn, NJ. Reagent grade Triethanolamine, (+)-catechin, NaCl, and maleic acid were purchased from Sigma-Aldrich, Inc., St. Louis, MO. Food grade Potassium bitartrate was purchased from Presque Isle Wine Cellars, North East, PA. Methacrylate 1.5 mL capacity cuvettes were purchased from Fisher Scientific.

3.2.4 Phenolics analysis

Methods

Analysis of wine samples for the content of phenolic groups was done based on the modified Adam's assay. The modified Harbertson-Adam's assay allows for measurement of all primary phenolic groups in wine (Harbertson *et al.,* 2002, Harbertson *et al.,* 2003). For tannins, it follows the procedure of Hagerman and Butler (1978) with the use of BSA protein in protein precipitation to determine tannin content of wine through use of ferric chloride (Harbertson *et al.,* 2002). One advantage of the method is the ability to determine polymeric pigment content of wine through the use of bisulfite bleaching which removes coloration of anthocyanin pigment but allows for coloration of polymeric pigments to remain. When this step is done along with the step of protein precipitation it allows for the size of polymeric pigments, large or small, to be determined (Harbertson *et al.,* 2003). The modified Adam's assay also accounts for the phenolic group of anthocyanins and provides a measure of total iron reactive phenolics in the wine samples. The assay has had widespread use in the wine industry (Landon *et al.,* 2008, Versari *et al.,* 2008).

Method Description

To measure iron reactive phenolics 725 µL of resuspension buffer was added to the 150 µL wine sample. The resuspension buffer made to a volume of 1 L was composed of 50 g of sodium dodecyl sulphate (SDS),50 mL triethanolamine, and the pH was balanced to pH 9.4 with HCl. Absorbance of the solution of wine and resuspension buffer in methacrylate 1.5 mL capacity cuvettes was measured at 510 nm wavelength using a spectrophotometer (Biomate 3, Thermo Scientific, Madison, WI). In order to get a total iron reactive phenolics reading the initial solution of wine and resuspension buffer had to have 125 µL of ferric chloride reagent added. The ferric chloride reagent made to 1 L fluid volume was composed of 2.7 g ferric chloride hexahydrate and 800 µL of 12.1 N, or 37%, HCl. The solution now containing ferric choride reagent was again measured at the 510 nm wavelength and the difference between the absorbance with and without ferric chloride was used to determine the total iron reactive phenolics. Blanks were composed of resuspension buffer for the solution of wine sample and resuspension buffer, and resuspension buffer and ferric chloride reagent for when ferric chloride had been added.

In order to assign a quantity to the amount of iron reactive phenolics and other phenolic groups measured by the modified Adam's assay, a standard curve was created for total iron reactive phenolics. The compound used for the standard was (+)-catechin. The standard was run using the same process as what was used for determination of the total iron reactive phenolics. Catechin was placed in 10% ethanol and ranged in concentrations of 0 mg/L to 300 mg/L.

Determination of polymeric pigments involved placing 200 µL wine samples and 300 µL of model wine solution into 1 mL of washing buffer and measuring absorbance at 520 nm. One liter of model wine solution is composed of 5 g potassium bitartrate, 120 mL of 96% ethanol and adjusted to pH 3.3 with HCl. A liter of washing buffer is made of 9.86 g of NaCl, 12 mL of glacial acetic acid and adjusted to pH 4.9 with NaOH. The second step of polymeric pigment measurement involves bleaching of the anthocyanins contained in the solution by use 120 µL of bleach solution. The bleach solution consisted of dissolving 2 g of potassium metabisulfite in 25 mL of Millipore water. The step

involving bleaching allows for a measurement of the coloration which polymeric pigments impart to the wine.

Measurement of tannins involved using a BSA protein solution and after adding 1 mL of the protein solution, and the 200 µL wine and 300 µL model wine components in a 2 mL eppendorf tube, the resulting solution was mixed to allow for precipitation of proteins by tannins. The protein solution was made by mixing of one mL of 40 mg/mL stock of bovine serum albumin (BSA) in 39 mL of washing buffer to arrive at protein solution having a concentration of 1 mg BSA/mL. The contents of the 2mL eppendorf tube were centrifuged to allow for formation of a pellet of precipitated protein. Use of 80 µL of bleach solution allowed for small polymeric pigments to be measured at 520 nm. Supernatant was removed. Then the addition of 500 µL of washing buffer removed any remaining phenolics not associated with the BSA protein. The pellet was resuspended in solution using 875 µL of the resuspension buffer to allow for measurement of tannins at 510 nm. Measurement at 510 nm of the resuspension with 125 µL of ferric chloride reagent addition allowed for tannins having reactivity to ferric chloride to be quantified.

Anthocyanins were measured using wine sample and model wine components and adding an anthocyanin buffer. The liter of anthocyanin buffer was composed of 23 g of maleic acid, 9.93 g NaCl, and adjusted to pH 1.8 with NaOH. The wine sample and model wine in the anthocyanin buffer in a cuvette was measured at 520 nm.

3.2.5 Tasting panel protocol

Member of the wine tasting panel in the spring of 2009 were selected based on their previous skill in tasting panels from the previous years of 2007 and 2008. It is of importance to have consistency with any tasting panel and this is something that was of particular challenge because of the nature of the wine tastings which allowed participants to join tastings after the series of tastings of the wines that were evaluated for their phenolic content had already begun. The amount of individual experience in wine tasting as well as an extended time in between each of the tastings created more subjectivity in the evaluations of the wines.

Therefore, only one individual tasting, which was later in the series of tastings in the spring of 2009, has been used to correlate phenolic groups with related tasting panel ratings. The later tasting panel in the series of tasting panels was used because members on the tasting panel had already experienced one or more previous tasting panels and had a greater inclination to evaluate the wines with more consistency.

As general practice at each tasting, panelists were given training wines at the beginning of the tasting to familiarize their pallet with intensity of wine attiributes such as astringency and mouthfeel. After taking a few moments to familiarize themselves, the panelists then began a series of wine flights.

A typical wine flight was composed of four wines and there were generally three to four flights at every tasting panel. Each wine sample was identified with a randomized number sticker previously assigned to the sample which was adhered to the foot of the wine glass, the part of the wine glass resting on the table. Panelists were given sufficient time to evaluate the wines and would indicate when they were through with one flight and ready to begin reporting their impressions on the next flight.

Panelists rated wines by giving their impressions about presence and intensity of particular characters of the wine. The scale chosen was a hedonistic scale with ten indicators of level of the character. As the numbers on the scale increased from left to right, this represented going from low to high intensity or bad to good. At the conclusion of every tasting panel, the panelist's wine evaluation hard copies were collected and kept for future reference and for use in stastical analysis of the wines.

3.2.6 Statistical analysis

SAS was the program used for the statistical analysis needed to generate results data (SAS Institute, Inc., Cary, NC, USA). The GLM procedure for unbalanced ANOVA was used to model data to determine affect of wine varietal. Significant difference in varietal means was determined by using an unequal N Tukey (Honestly Significant Difference) HSD test. The Tukey HSD test allows for uneven replicates to be used in obtaining the mean values of a given sample, normally consisting of three replicates. The event of

having uneven replicates was the case with replicates that were not able to be utilized from the analysis due to unavoidable lab errors, or in the event of having less than sufficient wine sample volumes to obtain three replicates per sample for every step of the modified Adam's assay.

3.3 Results

The tannin amount varied by the red wine varietals examined. The content of Chambourcin wine was lowest of the five red wine varietals. The tannin content of Merlot wine was highest of the five varietals. It is evident in Table 3.1 that the varietals have significantly different means at the $p \leq 0.05$ level.

The anthocyanin content varied with wine varietal (Table 3.1). The lowest mean anthocyanin content of the five varietals was in the varietal Cabernet Franc which had a content of 87.38 ± 4.05 mgL^{-1} anthocyanin. The average anthocyanin of Norton wine was 546.6 ± 3.26 mgL^{-1} anthocyanin, representing the highest anthocyanin content of the varietals.

Table 3.1. Phenolic content of five varietal's wines expressed as cultivar means ± standard error

Cultivar	Tannin (mgL^{-1} CE)	Anthocyanin (mgL^{-1} Anthocyanin)	LPP (absorbance)	SPP (absorbance)	Iron-reactive phenolics (mgL^{-1} CE)
Cabernet Franc	278.4 ± 11.09 c	87.38 ± 4.05 d	1.03 ± 0.062 c	1.46 ± 0.066 d	635.3 ± 14.50 b
Cabernet Sauvignon	455.1 ± 7.53 b	166.3 ± 2.42 c	1.63 ± 0.042 a	2.16 ± 0.045 c	976.3 ± 8.74 a
Chambourcin	42.55 ± 10.08 e	399.5 ± 3.49 b	0.36 ± 0.059 d	2.49 ± 0.060 b	451.1 ± 12.25 c
Merlot	530.5 ± 13.53 a	173.5 ± 4.53 c	1.30 ± 0.076 b	1.43 ± 0.081 d	999.1 ± 16.21 a
Norton	104.9 ± 9.45 d	546.6 ± 3.26 a	0.84 ± 0.055 c	2.99 ± 0.056 a	659.9 ± 11.46 b

Table 3.1 (Continued). Phenolic content of five varietal's wines expressed as cultivar means ± standard error

Number of wines sampled are Cabernet Franc (n=10), Cabernet Sauvignon (n=28), Chambourcin (n=14), Merlot (n=8), and Norton (n=16). Lowercase letters (a-e, a being the highest rank) within the table denote statistically significant differences ($p \leq 0.05$) using an unequal N Tukey Honestly Significant Difference (HSD) test.

Absorbance due to polymeric pigments in the wine also differed with respect to varietal for wines. For large polymeric pigments absorbance readings were measured at 520 nm for this group of phenolics. There was no significant difference in the Cabernet Franc and Norton varietals when using Tukey's HSD (Honestly Significant Difference) test to compare absorbance at 520 nm due to LPP of the varietals at the $p \leq 0.05$ level (Table 3.1). All of the remaining varietals did have significant difference in their mean absorbance at 520 nm due to LPP.

For small polymeric pigments, the absorbance was read 520 nm as part of quantification of this group of phenolics. There was no significant difference in the Cabernet Franc and Merlot varietals when using Tukey's HSD (Honestly Significant Difference) test to compare absorbance at 520 nm due to SPP of the varietals at the $p \leq 0.05$ level (Table 3.1). All of the remaining varietals did have significant difference in their mean absorbance at 520 nm due to SPP.

Total phenolics were a group with considerable differences among certain varietals while other varietals were rather closely matched in the iron-reactive phenolics content. The same units of phenolic content mgL^{-1} CE were used for quantifying the level of iron-reactive phenolics. There was no significant difference with two pairs of the varietals. There was no significant difference in means of the Merlot and Cabernet Sauvignon varietals and there was no significant difference in means of the Norton and Cabernet Franc varietals when using Tukey's HSD (Honestly Significant Difference) test to compare iron-reactive phenolic content of the varietals at the $p \leq 0.05$ level (Table 3.1).

Chambourcin's phenolic content was different than the contents of the other four varietals (Table 3.1).

When red wines that had been analyzed for phenolic content were compared to tasting panel data there were strong correlations. The tasting descriptors of astringency rating, color rating, and overall acceptability were all strongly correlated with phenolic group content of the wines. The strongest correlation of any tasting panel evaluation criteria was astringency rating correlated with the tannin content of the wines with an R of 0.9111(Figure 3.1 A). The second strongest correlation of R=0.7322 was with color rating and anthocyanin content of the wines (Figure 3.1 B). Overall acceptability rating and total iron-reactive phenolics were correlated with the R=0.7026 (Figure 3.1 C). When used in combination, tasting panel ratings can help to decide if a wine is balanced in its level of phenolic groups or whether one or more of these groups is unbalanced. In particular, the distribution of iron-reactive phenolics content of wines, see Figure 3.1 C, lends support to the idea of a wine needing to be moderate in its phenolic content in order to be deemed acceptable in its taste.

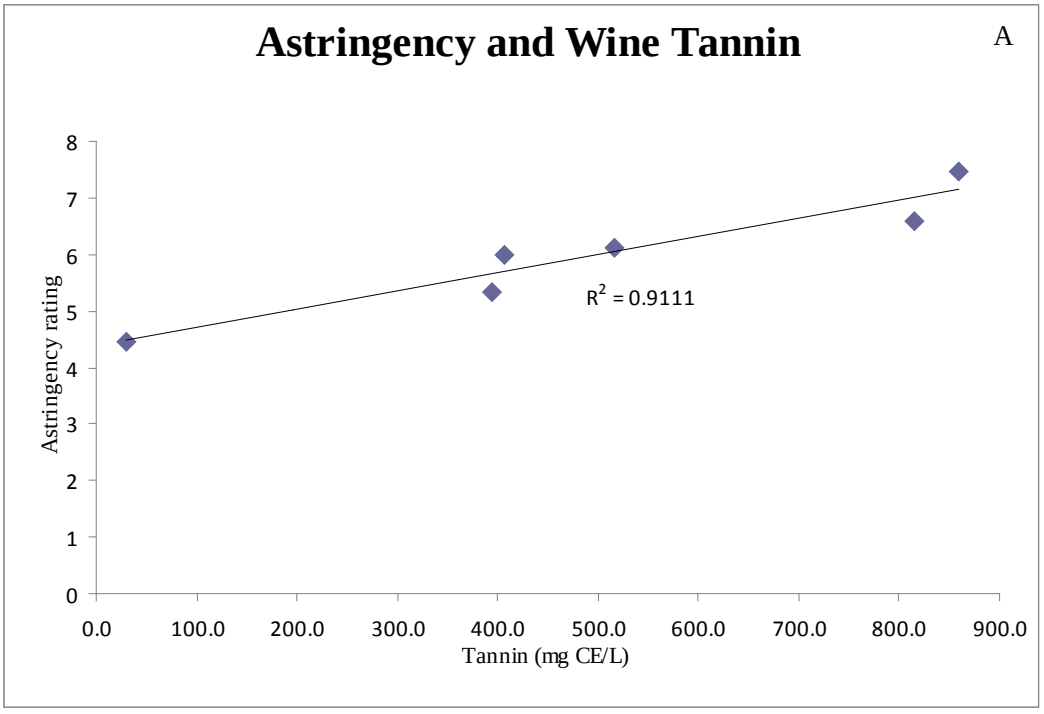
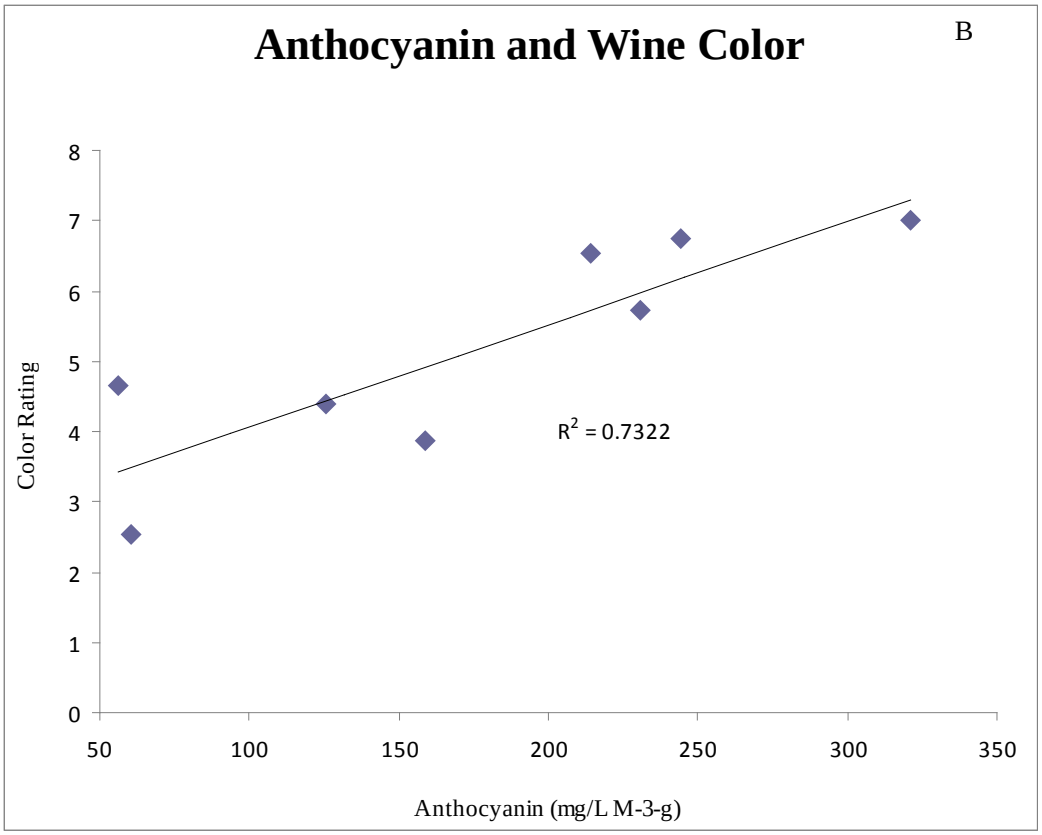


Figure 3.1


B
Anthocyanin and Wine Color
Color Rating
Anthocyanin (mg/L M-3-g)
R2 = 0.7322
0
1
2
3
4
5
6
7
8
50
100
150
200
250
300
350

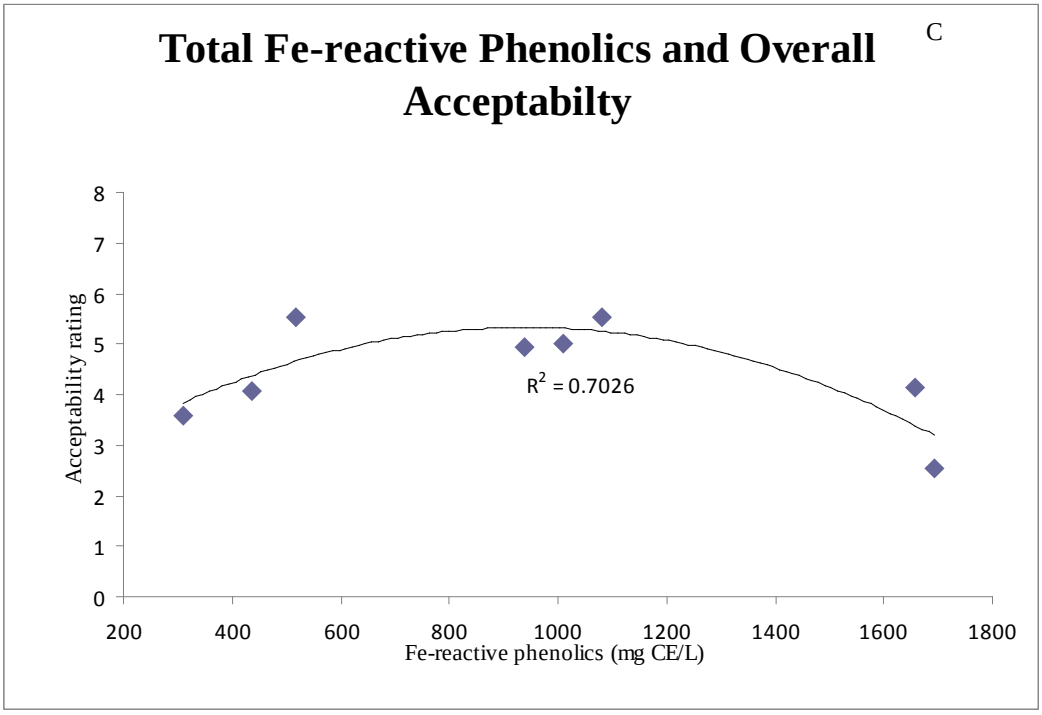

C
Total Fe-reactive Phenolics and Overall Acceptabilty
Acceptability rating
Fe-reactive phenolics (mg CE/L)
R2 = 0.7026
0
1
2
3
4
5
6
7
8
200
400
600
800
1000
1200
1400
1600
1800

Figure 3.1

Figure 3.1 Correlations between phenolic group content of red wines and the respective average score that each red wine received by the tasting panel (A), tannin content in mg catechin equivalents per liter of wine (mg CE/L) correlated with astringency rating (B), anthocyanin content in mg Malvidin-3-glucoside per liter of wine (mg M-3-g/L) correlated with color rating (C), iron-reactive phenolics content in mg CE/L correlated with overall acceptability rating.

3.4 Discussion

Regarding the phenolic content, the balance that exists between tannins and anthocyanins resulted in high correlations with the tasting descriptions of proper astringency, mellowness, and balance. The result of the research that dealt with phenolic composition and flavor of red wines was that wines that possess higher levels of anthocyanins and a small amount of tannin should be of good quality. The use of oak to boost phenolic levels and increase mouthfeel has been done for a multitude of wines. A common method of producing Chambourcin wine has been using oak barrels to age the wine prior to its release to consumers. One such occurrence that is not necessarily intended or even completely understood is how exactly oak ellagitannins reduce wine oxidation while aging wines (Vivas and Glories, 1996).

The presence of salivary proline-rich proteins (PRPs) which are highly affective at creating insoluble bonding with tannins are what form the distinctive astringent sensation commonly experienced when having a glass of wine (Lu and Bennick, 1998). The astringency is the result of protein precipitation in the presence of the tannins in the wine. Skin tannin of grapes is also considered to have a more mellow mouthfeel than the tannin counterpart found in seeds, and luckily the tannin extraction from grape seeds is low (Cheynier *et al.*, 1998). Astrigency differs in tannins based on the length of the tannin polymers, with the less astringent grape skins clearly having a greater molar ratio of total units to end units than the tannins found in the grape seeds (Vidal *et al.*, 2002). Additionally, it is the composition of the subunits which compose the tannins in both the seeds and skins. The balance of epicatechin to catechin subunits is a determinant in the

perceived organoleptic experience of wine tannins. The catechin and epicatechin subunits of seed tannins are more balanced and give a bitter nature to the wine while the skin tannins have more catechin subunits than epicatechin subunits and impart primarily an astringent nature to the wine (Thorngate and Noble, 1995). The level of protein precipitation of tannins is influenced by the amount of polymerization that wines have undergone. The greater the level of polymerization the less astringent the wine will seem when doing sensory analysis of the wine (Kennedy *et al.*, 2006). Tannins accumulate mostly before verasion in the grape, while anthocyanins accumulate post-veraison in the grape and both are important indicators in grape maturity and are vital in wine production (Downey *et al.*, 2003a).

Anthocyanin content varied noticeably among the varietals used to make their respective wines. There was a very striking difference between the mean anthocyanin content of Cabernet Franc and Norton (Table 3.1). Basis for such difference when excluding wine production methods includes genetic differences among the cultivars. The observation of gene expression specific to the enzymes responsible for berry anthocyanin accumulation means that any variation which exists between the cultivars in regard to the degree of expression of these particular genes would allow for varying levels of anthocyanin accumulation based on cultivar (Boss *et al.*, 1996b). Additionally, the cultivar performance is a determinant in cultivar capability in anthocyanin accumulation in a given growing region. Cultivar performance is defined by the ability of adequate coloration to be achieved under climatic conditions including high temperatures. Cultivars having the highest anthocyanin concentration in grape clusters were associated with better cultivar performance in high temperatures (Kliewer and Torres, 1972).

Stability of anthocyanin contents later in berry maturity for Cabernet Franc, Merlot, and Pinot Noir has been discussed in previous study, and may explain differences in the mean contents produced by the varietals in this study (Mazza *et al.*, 1999). Such observation in stability of anthocyanin content may explain why such a tier exists between the varietals of Cabernet Franc, Cabernet Sauvignon, and Merlot which were all below contents of 200 mg anthocyanin L^{-1}, and Chambourcin with a mean of approximately 400 mg anthocyanin L^{-1} and Norton with its mean of approximately 550 mg anthocyanin L^{-1}

(Table 3.1). Cultivars may display differences in amount and duration of gene expression for anthocyanin accumulation in addition to mechanisms existing in cultivars such as Chambourcin and Norton that appear to have better cultivar performance in this region than the cultivars of Cabernet Franc, Cabernet Sauvignon, and Merlot. Support for cultivar performance was found in a study examining wines from British Columbia which had been produced in different locations in the same growing appellation, yet noticeable differences in coloration of wines of a given cultivar suggests that localized climates and suitability of a given cultivar is one determinant of its wine coloration (Cliff *et al.*, 2007).

Additionally, an explanation as to why particular wines maintain stable coloration as wines made from some cultivars age. The degree polymerization allows for wine coloration to become stable due to the coloration provided by the polymeric pigments (Somers, 1971). The degree of polymerization is due to amount of anthocyanin content and presence of flavonols in the wine. Table 3.1 shows the contents for total phenolics in Norton wine and Cabernet wine were moderate for all five varietals in this study while the total phenolics in Cabernet Sauvignon wine and Merlot wine were high when considering the measurements of the five varietals in this study. Low polymerization causes wines to display low coloration after being produced. Wine made of dark cultivars such as Pinot Noir or Sangiovese have low cofactor contents and display very little polymerization which results in the bright red coloration in young wines made of these particular cultivars. Wines like Pinot Noir and Sangiovese which have very low levels of polymeric pigments can be easily determined by use of bisulfate bleaching to remove coloration solely due to anthocyanins and determine amount of coloration attributed to polymeric pigments (Boulton, 2001). Such method of separation of the color fractions due to anthocyanins and the ones due to polymeric pigments was achieved in part by the use of bisulfate bleaching in our study.

3.5 Conclusion

The contents of phenolics were found to have a strong association to the cultivars from which they were made. The low standard errors for contents of tannins, anthocyanins, SPP and LPP, and total phenolics support the arbitrary association that can be made between the cultivar and the measured phenolic composition in wine (Table 3.1). Further support to this claim lies in Table 3.1 which shows varietal stratification in the means of phenolic contents for the Cabernet Franc, Cabernet Sauvignon, Chambourcin, Merlot, and Norton varietals that were included in this study. To include additional support, the wines at an individual wine tasting held to compare the sensorial quantifications to the experimentally derived quantifications showed strong linkage with astringency and wine tannin (R=0.91), anthocyanins and wine color (R=0.73), and total Fe-reactive phenolics and overall acceptance level (R=0.70). Graphs having individual wines consisting of three varietals and showing the correlations are in Figure 3.1.

In conclusion, the idea that multiple phenolic compounds in a wine such as tannins, anthocyanins, total Fe-reactive phenolics and SPP and LPP formed by polymerization of wine phenolics can adequately be connected to a cultivar has been supported in this study. The varietals did have commonality with some of the individual phenolic groups, such as the anthocyanin contents of Cabernet Sauvignon and Merlot, or the total Fe-reactive phenolics of Cabernet Franc and Norton varietals and those of Cabernet Sauvignon and Merlot; however, upon examining all five of the phenolic groups quantified in this study, it is clear that no two varietals were exactly alike. Each varietal is unique in its overall profile. The bar chart of Figure 3.2 demonstrates the noticeable differences of all five varietals when looking at means of tannins, anthocyanins, and total Fe-reactive phenolics.

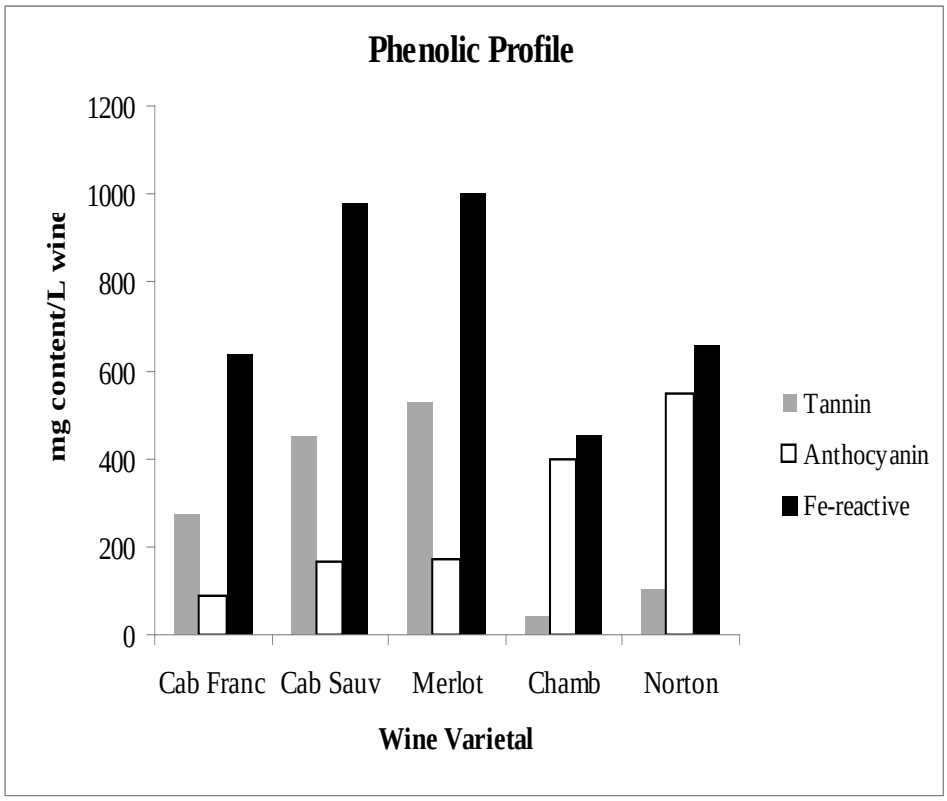


Figure 3.2 Bar chart illustrating the differences among varietals between multiple phenolic groups for the five cultivars that included Cabernet Franc, Cabernet Sauvignon, Chambourcin, Merlot, and Norton. The varietals have been arranged in ascending order of their mean anthocyanin content (mg/L of anthocyanins) on the bar chart. The mean contents of two other groups were examined: Fe-reactive phenolics (mg CE/L) and tannin (mg CE/L).

CHAPTER 4: CULTIVAR STUDY

4.1 Introduction

Analysis of compounds in wine grapes that are of importance in wine is commonplace. Generally the most widely grown wine grape cultivars are examined such as the Cabernet Sauvignon cultivar (Cliff *et al.,* 2007; Deluc *et al.,* 2007; Harbertson *et al.,* 2008; Landon *et al.,* 2008).

Examination of levels of phenolics versus grape cultivar has been researched because of an interest as to whether the cultivar or in similar terms, the genetics, plays a role in accumulation of the phenolic compounds in grapes. There are numerous papers which have investigated the variability in red wine grape cultivars and their phenolic composition (Mazza *et al.,* 1999; Arozarena *et al.,* 2002; Ryan and Revilla, 2003; Munoz-Espada *et al.,* 2004).

The organic acid content in grapes has also been compared among cultivars. Grapes vary in their ability to accumulate tartaric acid with one species known to contain undetectable amounts of tartaric acid which is usually the primary organic acid in grapes. The species *Ampelopsis aconitifolia* both contains undetectable levels of tartaric acid and has ascorbic acid content that is much higher than found in species which have TA as the abundant organic acid. An explanation for such contrasting patterns of accumulation can be found in the fact that a gene controlling tartaric acid biosynthesis from ascorbic acid is not present in *Ampelopsis aconitifolia* (DeBolt *et al.,* 2006).

There is a great potential for variation in the berry phenolics content of cultivars of red wine grapes (Mazza *et al.,* 1999; Arozarena *et al.,* 2002; Ryan and Revilla, 2003; Munoz-Espada *et al.,* 2004). The same is true for organic acids where contents of individual organic acids can be multiple factors of difference. A good example is the case of tartaric acid which reached a high of 15 mg per gram of berries fresh weight in one species to a low of <1.5 mg per gram fresh weight in three of twenty-eight species examined (DeBolt *et al.,* 2006). In a comparison of two wine grape cultivars, shiraz and semillion, both had insignificant differences in tartaric, malic, and oxalic acids, the major organic acids at

harvest, but when comparing the two there were significant differences of individual organic acids during growth stages preceding the harvest date (Melino *et al.*, 2009).

The benefit of a time-lapse approach, or in other words sampling at multiple stages points during the growing season, in determination of the important phenolics and organic acids in grapes is that trends in their accumulation over time can allow for a better understanding of the ideal habitats that cultivars may have at certain developmental stages. A particular cultivar may be more heat-sensitive, and through high respiration rates, unable to sustain the optimal levels of phenolics like anthocyanins (Kliewer and Torres, 1972). The same sort of rationale applies to accumulation ability of organic acids like malic acid which is known to be higher in vines of wine grapes that are grown in cooler regions (Conde *et al.*, 2007). Enzymatic activity regulating high malic acid accumulation is optimal at temperatures ranging 20°C to 25°C (Lakso and Kliewer, 1975).

It is quite certain that a combination of genetic and environmental effects influence the way grapes accumulate the phenolics and organic acids important for winemaking. Looking at the berry content of phenolic compounds and organic acids throughout the growing season can allow for trends of accumulation to be noticed. Sampling at regular intervals post-flowering gives the potential for distinctions to be made based on grape cultivar.

4.2 Materials and Methods

4.2.1 Chemicals and standards

Buffer Solution pH 7.00 for use in berry parameter analysis, reagent grade NaOH and HCl, and phosphoric acid for use in organic acid extraction were was purchased from Fisher Scientific, Fair Lawn, NJ. Methanol used in phenolics extraction was purchased from Fisher Scientific, Trinidad. Chlorogenic acid to be used for the phenolic's standard, and HPLC-grade potassium phosphate monobasic for organic acid analysis by HPLC were purchased from Sigma-Aldrich, Inc., St. Louis, MO.

4.2.2 Plant material

Berry sampling of two *Vitis vinifera* L. cultivars Cabernet Franc and Cabernet Sauvignon and two French-American hybrid cultivars Chambourcin and Norton was done over two seasons. The grapevines were located in a vineyard at the University of Kentucky Horticulture Research Farm Lexington, Kentucky, USA. The particular section of the vineyard used for berry sampling was established in 2006 with 2.5 m vine spacing and 2.8 m row spacing. In both years of study, vines were pruned by hand to 40 to 50 nodes per vine. No other vine management techniques were practiced besides shoot thinning to 3 to 5 nodes per foot of cordon.

A total of 16 rows composed the plot. In the plot, cultivars were arranged in a generalized random block design. Two vines of a cultivar were adjacent in a row and the cultivar grouping was repeated in three random places to give six total vines of a cultivar in two rows. Twelve vines of each cultivar were used for the analysis of the berries. Three replicates were done for each cultivar by combining individual clusters of four vines per sample. Grape berries were first sampled at capfall, to henceforth be referred to as the end of flowering. The end of flowering for the 2009 season happened as follows: May 28th for Chambourcin, June 1st for Cabernet Franc, June 3rd for Cabernet Sauvignon, and June 5th for Norton. In 2010, all of the four cultivars attained the end of flowering on June 3rd. Every two weeks post-flowering grape clusters were collected. Collections continued until harvest as determined by the berry parameters analysis that measured pH, brix, and TA to arrive at ideal maturity levels was at times 16 to 18 weeks post-flowering. Such ideal maturity levels included pH < 3.7, brix above 20° - ideally 22°-25° Brix, and TA of approximately 7 g per liter of berry juice, or approximately 0.65% content of the berry. These criteria are very much in agreement with the Midwest Grape Production Guide Bulletin which references common criteria for evaluating maturity and ranges that typically indicate maturity for red wine grapes used to make dry red wines (Dami *et al.*, 2005).

The clusters which were collected at the vineyard were then taken back to the lab at the Agricultural Science Center North at the University of Kentucky. One half of the

replicate was set aside for pH, titratable acidity, and brix analysis to be done right after the collection arrived at the lab. The remaining portion of the replicate was frozen in liquid nitrogen and stored in a freezer at -20°C for berry phenolics analysis and organic acid analysis.

4.2.3 Berry parameters analysis (pH, titratable acidity, and brix)

Analysis of pH was done by using a pH/mV/°C meter, pH 510 series, Malaysia. The meter was pre-calibrated before use to pH 7.00 with a pH 7.00 buffer solution. For use in the pH measurement of replicates, and when measuring titratable acidity, and brix, the portion of the replicate that was set aside for berry parameters analysis was placed into a sealable plastic bag and the berries were crushed thoroughly.

A small-sized 40 mL beaker was used to hold the juice produced from crushing of the berries in the sealable plastic bag. The pH meter was turned on and thoroughly rinsed before using it to measure the pH of the sample. Measurement involved placing the pH meter directly into the grape juice in the 40 mL beaker. The pH of all three replicates was measured.

After measurement of the pH, titratable acidity was measured. Measurement of titratable acidity followed the method described in Cottrell (1968). The contents of the 40 mL sample beaker were mixed before pouring 10 mL of the juice into a 20 mL graduated cylinder. The volume of 10 mL of sample grape juice was poured into a 125 mL Erlenmeyer flask containing 100 mL of Millipore water. The pH meter was turned on for the duration of the procedure. Using 0.1 N NaOH, the pH was adjusted to an endpoint of 8.2. A 1µL-1,000µL pipette was used to deliver the 0.1 N NaOH into the Erlenmeyer flask. Accuracy was done to the nearest 25 µL of 0.1 N NaOH. The titratable acidity was expressed as grams of titratable acids per liter of juice. Three replicates for every cultivar were done for measurement of titratable acidity.

When complete with titratable acidity measurements, brix of the sample was measured. Measurement of brix used a refractometer (Reichert Scientific Instruments, Buffalo, NY). The refractometer was precalibrated before use. The contents of the sample beaker were

thoroughly mixed before applying a drop-sized amount of the grape juice to the prism of the refractometer using a thin plastic bar included with the refractometer. Measurement was done by gazing through the eyepiece into a bright light and estimating the degree brix to the nearest tenth of a degree. Three replicates of the degree brix measurement were done for every cultivar.

4.2.4 Phenolics analysis

Extraction of grape berry phenolics

Phenolics were extracted from the three replicates of each of the four cultivars for every collection. The Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton samples were taken from storage at -20°C.

Samples were prepared in 80% methanol as described in the method by Fukumoto and Mazza (2000) for phenolics analysis. One gram of samples collected at the end of flowering was used. Ten grams of samples collected at two weeks post-flowering was used. For samples four weeks post-flowering through harvest (eighteen weeks post-flowering), 30 g of sample was used. The samples were placed in a waring blender with 40 mL of 80% methanol and mixed for eight minutes at low speed. The samples were then filtered using a Whatman #42 filter paper placed in a 1 L Erlenmeyer vacuum flask containing a porcelain funnel connected to a vacuum.

Measurement of grape berry phenolics

Phenolics analysis followed the modified Glories' method procedure in Fukumoto and Mazza (2000). The following steps were done for the phenolics analysis:

1. Dilution of extracted phenolics samples with 5% Methanol to achieve spectrophotomeric readings in the 0.1 to 1 Absorbance range. For 30 g fresh-weight berry samples this was achieved with a dilution factor of 5 or 6.667.

2. Placing 0.25 mL of sample or standard in a small beaker and adding 0.25 mL of 0.1% HCl in 95% ethanol and 4.55 mL of 2% HCl.

3. The solution is mixed and allowed to sit for 15 minutes before reading the absorbance at 280 and 520 nm with a spectrophotometer by placing the solution from the beaker into a 3 mL glass cuvette (Fisher Scientific). Three replicates were done for each of the samples. The absorbance at 280 nm corresponds to the total phenolics content of the sample. The absorbance at 520 nm is used as the anthocyanin content estimate.

4. Standards included chlorogenic acid in 80% MeOH for the total phenolics. The anthocyanin content was obtained by using the anthocyanin extinction constant for Malvin-3-glucoside which has been reported as 28000 $L \cdot cm^{-1}mol^{-1}$which has been computed using Beer's Law.

4.2.5 Organic acids analysis

Extraction of organic acids

The procedure followed the method described by DeBolt *et al.* (2004). The berries, which had been in the collection bags in a -20°C freezer, were weighed to obtain approximately 5 g fresh weight of berries for each of the three replicates and the berry number and actual mass to milligram accuracy were recorded. This was ground by mortar and pestle using 5 mL of 0.5 M H_3PO_4 at pH 1.5. The contents of the mortar were transferred to a 2 mL centrifuge tube that was used to hold the slurry. The centrifuge tube was placed onto a rotating mixer for 2 hours to thoroughly protonate the slurry. After the slurry was mixed, the 2 mL microcentrifuge tube was rotated at 14,000 rpm for 3.5 minutes by centrifuge. The spun aliquot was then passed through a syringe containing a 0.45 µm filter (0.45 µm millex-HN syringe driven filter unit, Millipore Corporation) before being delivered by pipette to the glass vials to be placed onto the HPLC autosampler.

Measurement of the organic acids

The organic acid extracts in the glass HPLC vials were placed on forty-well HPLC autosampler trays and run on the HPLC autosampler (Dionex, Ultimate 3000). The column (Prevail organic acid 4.6 x 150 mm, Grace Davidson Discovery Sciences) was maintained at 30°C with an injection temperature of 25°C. Injection volume was 10µL. The mobile phase used in the reversed-phase HPLC analysis was 2.5 mM KH_2PO_4 that had been adjusted to pH 2.5 using H_3PO_4. Flow rate was 1mL of mobile phase per minute. Detection in order of elution of oxalic (OA), tartaric (TA), malic (MA), and citric (CA) acids was using a diode array detector with UV absorbance at 210 nm.

4.2.6 Statistical analysis

The R version 2.9.2 statistical computing program was used in the cultivar study (R 2.9.2, Vienna, Austria). In addition to running code for means, and standard errors for phenolic, anthocyanin, organic acids, and berry parameters data, the program was also used to create models of climatic data to test for the significance of these factors. The ANOVA summary models of the climatic data used the temperature threshold allowing a 20° C maximum and year as the two climatic factors investigated. Temperature threshold in this study is defined by the number of days up to the date of collection in which the minimum temperature of 20° C was either met or below this number of degrees Celsius. For temperature threshold in 2009 and 2010, see Table 4.1. The value of 20° C for the temperature threshold was based on the controlled temperature study of Kliewer and Torres (1972), in which cool season cultivars were unable to accumulate their ideal anthocyanin contents if night-time temperatures were above 20° C. Cultivars which accumulated anthocyanins in higher temperatures, 30° C, for example, did not experience loss of ability to accumulate anthocyanins which allows separation between cultivars on basis of the factor temperature threshold. For the entire two year collection of the four cultivars, phenolics and associated berry parameters data and organic acids and associated berry parameters data were used to create ANOVA summary models of temperature threshold by cultivar and year by cultivar. For individual cultivars, both temperature threshold and year ANOVA summary models were created to determine significance of these factors on individual cultivar phenolics and associated berry parameters data and organic acids and associated berry parameters data.

4.3 Results

4.3.1 Phenolics analysis

In analysis of variance for the entire collection of all four cultivars, the interaction between cultivar and temperature threshold was examined for significance ($p \leq 0.05$). Temperature threshold in this study is defined by the number of days up to the date of

collection in which a minimum temperature of 20° C was either met or below this number of degrees Celsius, for temperature threshold in 2009 and 2010 see Table 4.1. Testing the cultivar and year interaction for significance ($p \leq 0.05$) was also performed with the years being 2009 and 2010. For cultivar phenolics, (SEE APPENDICES; Appendix A.4: Wine Phenolics, Table A.4.1), there was significance of the interaction of temperature threshold ($p = < 0.001$) and the cultivar by temperature threshold interaction ($p = 0.002$). Additionally the interaction of year on phenolic content was also significant with a p-value of 0.025. Anthocyanin content, (SEE APPENDICES; Appendix A.4: Wine Phenolics, Table A.4.1), was highly significant with cultivar and temperature threshold interactions. Cultivar, temperature threshold, and cultivar by temperature threshold interaction were all significant for the anthocyanin content of the grapes with all of their p-values < 0.001. Also, year had significance on anthocyanin content, thus year was significant for anthocyanin accumulation ($p = 0.031$).

Table 4.1. Temperature thresholds for cultivar collection

collection (days after flowering)		temperature threshold[a]	
		2009	2010
2009	2010		
0	0	0	0
14	14	15	13
28	28	22	17
42	42	33	26
56	56	45	30
70	70	57	32
84	84	66	38
98	98	80	50
112	109* CH	94	60*
118* NT	112** CF & NT	102*	63**
120* CS	119* CS	102*	69*
122* CF		103*	
126* CH		108*	

Table 4.1 (Continued). Temperature thresholds for cultivar collection

[a] Number of days in which the mininum temperature was at or below 20° C. The number is representative of the total applicable days this threshold was attained leading up to the collection. * represents collection in which grapes were harvested. The cultivar abbreviation is included under collection for more clarity with the cultivar abbreviated as Cabernet Franc (CF), Cabernet Sauvignon (CS), Chambourcin (CH), Norton (NT). ** denotes that this is harvest collection for CF and NT. CS was collected but no harvested at that collection. Data was utilized from the University of Kentucky Agricultural Information Center to form number of temperature threshold days.

Investigation of the significance of cultivar and temperature threshold on berry mass, (SEE APPENDICES; Appendix A.4 Wine Phenolics, Table A.4.1), of the entire collection revealed that the cultivar ($p < 0.001$), temperature threshold ($p < 0.001$), and cultivar by temperature interaction ($p = 0.002$) were all significant.

Each cultivar was separated looked at to determine the significance ($p \leq 0.05$) of temperature threshold and year on the phenolic content, the anthocyanin content, and the berry mass; the total attained number of days of temperature threshold for each cultivar is found in Table 4.1. For Chambourcin, the temperature threshold was significant for the phenolic content ($p < 0.001$). Temperature threshold had significance on anthocyanin content ($p < 0.001$). For berry mass of Chambourcin, the temperature threshold was highly significant ($p < 0.001$). Year had no significance on phenolic content, anthocyanin content, and berry mass of Chambourcin.

In Cabernet Franc, the factors of temperature threshold ($p < 0.001$) and year ($p = 0.039$) were both significant to the phenolic content. The same was true for anthocyanin content, with temperature threshold having a p-value of < 0.001, and year having a p-value of 0.0381). The factor temperature threshold was significant for berry mass of Cabernet Franc ($p < 0.001$).

In Cabernet Sauvignon, temperature threshold ($p < 0.001$) and year ($p = 0.038$) both had significance on the anthocyanin content. Temperature threshold had significance on the phenolic content ($p = 0.039$) and berry mass ($p < 0.001$) of Cabernet Sauvignon grapes.

In Norton, temperature threshold was highly significant for anthocyanin content ($p < 0.001$). The factor temperature threshold was also highly significant for berry mass of Norton ($p < 0.001$).

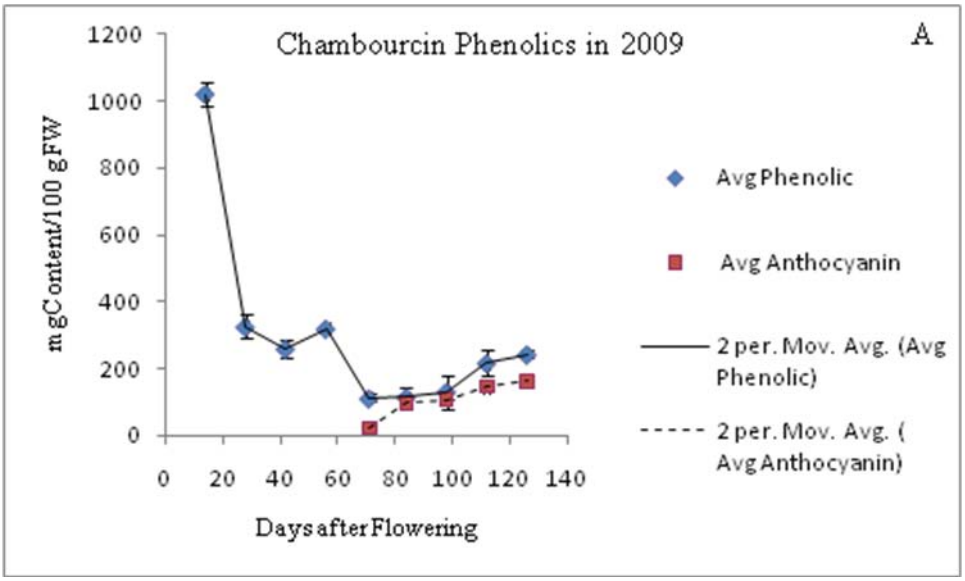
Phenolic content in the grape growing season of 2009 was sigmoidal in how the content, presented in Figure 4.1, changed with days post-flower (days after flowering) these results represented in Figure 4.1. All of the cultivars saw a substantial increase at 56 days (8 weeks) after flowering. This might be considered an indication of "priming" of the phenylpropanoid biosynthetic pathway for flavanoid production and makes sense because of the fact that anthocyanin accumulation was evident starting at 10 weeks for all cultivars with the exception of noticeable anthocyanin content in Cabernet Sauvignon at 8 weeks. Furthermore, phenolic content of the grapes was at its highest 42 to 70 days after flowering when evaluating content from the time period of 42 to 70 days post-flower until the harvest date at approximately 18 weeks after flowering. The one exception of the phenolic high at 8 weeks when looking at the time of 8 weeks until harvest was Norton (Figure 4.1). For Norton, an initial decline after the 8 week collection was noted, but unlike the other cultivars which did not substantially accumulate phenolics after the decline, Norton had its highest phenolic level for the period of 8 weeks until harvest occurring at the 16 week after flowering collection. The substantial increase in the time period of 8 weeks to 16 weeks after flowering for phenolic content for Norton indicates that the phenylpropanoid pathway for phenolic accumulation is more active in comparison to the other cultivars which show minor decline in phenolic content from 8 weeks until harvest.

Phenolic content in 2010 had similar accumulation pattern with its cultivars (Figure 4.2). Like in the previous growing season of 2009, cultivars saw substantial increase with phenolic content at the collection 56 days (8 weeks) after flowering. The increase corresponds with the first anthocyanin readings measured at the time of veraison. The

start of anthocyanin accumulation began at 8 weeks after flowering for Cabernet Franc, 10 weeks after flowering for Chambourcin and Norton and 12 weeks after flowering for Cabernet Sauvignon. Decline in phenolic content in the grape cultivars began when anthocyanin accumulation began. This would mean the phenolic contents were highest at the collection at 8 weeks after flowering when considering the time period from 8 weeks until the harvest collections at 16 weeks for Cabernet Franc, Chambourcin, and Norton, and 17 weeks for Cabernet Sauvignon. In 2010, the cultivar Chambourcin exhibited phenolic increase after the collection at 8 weeks after flowering, in fact, the highest phenolic content for the second half of the growing season was at 16 weeks. Previously, in 2009, Norton had the highest phenolic content at 16 weeks after flowering for the time from 8 weeks after flowering until the harvest collection at 18 weeks. Chambourcin saw an increase in phenolic content from the collection at 8 weeks after flowering until the harvest collection at 16 weeks after flowering. The cultivar Cabernet Franc had substantial decrease of its phenolic content, the cultivar Cabernet Sauvingon also experienced decreases in its phenolic content, and the cultivar Norton also had decline in its phenolic content all changes for the same time period as the phenolic increase for Chambourcin reported above.

Anthocyanin content expressed as mg malvidin-3-glucoside (m-3-g) per 100 g fresh weight (FW) of berries was similar in starting of accumulation for all cultivars in 2009 (Figure 4.1). Detection of pigmentation at the 520 nm wavelength took place at 0 days after flowering, when there were still reminants of flower parts on the grape samples, but this would be pigmentation due to flowering or even pollen, so little importance of the anthocyanin content should be placed on the collection at 0 days after flowering or at 14 days after flowering as observed with Chambourcin (Figure 4.1).

Anthocyanin accumulation followed similar patterns of accumulation in 2010, see Figure 4.2. Both Chambourcin and Norton began anthocyanin accumulation at 10 weeks after flowering. Cabernet Franc began anthocyanin accumulation at 8 weeks after flowering, while Cabernet Sauvignon anthocyanin accumulation did not begin until 12 weeks after flowering.


Chambourcin Phenolics in 2009
A
mg Content/100 g FW
1200
1000
800
600
400
200
0
0 20 40 60 80 100 120 140
Days after Flowering
Avg Phenolic
Avg Anthocyanin
2 per. Mov. Avg. (Avg Phenolic)
2 per. Mov. Avg. (Avg Anthocyanin)

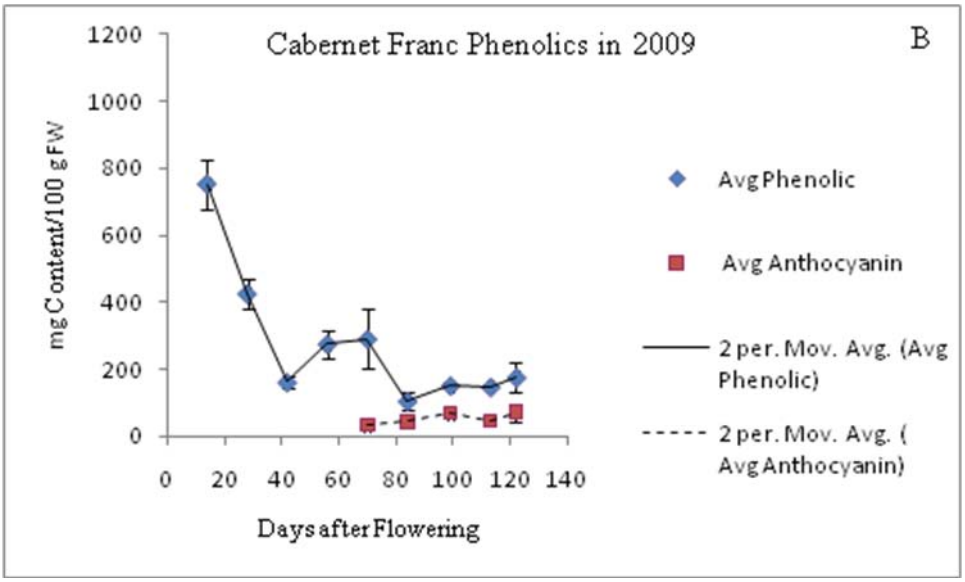

Cabernet Franc Phenolics in 2009
B
mg Content/100 g FW
1200
1000
800
600
400
200
0
0 20 40 60 80 100 120 140
Days after Flowering
Avg Phenolic
Avg Anthocyanin
2 per. Mov. Avg. (Avg Phenolic)
2 per. Mov. Avg. (Avg Anthocyanin)

Figure 4.1

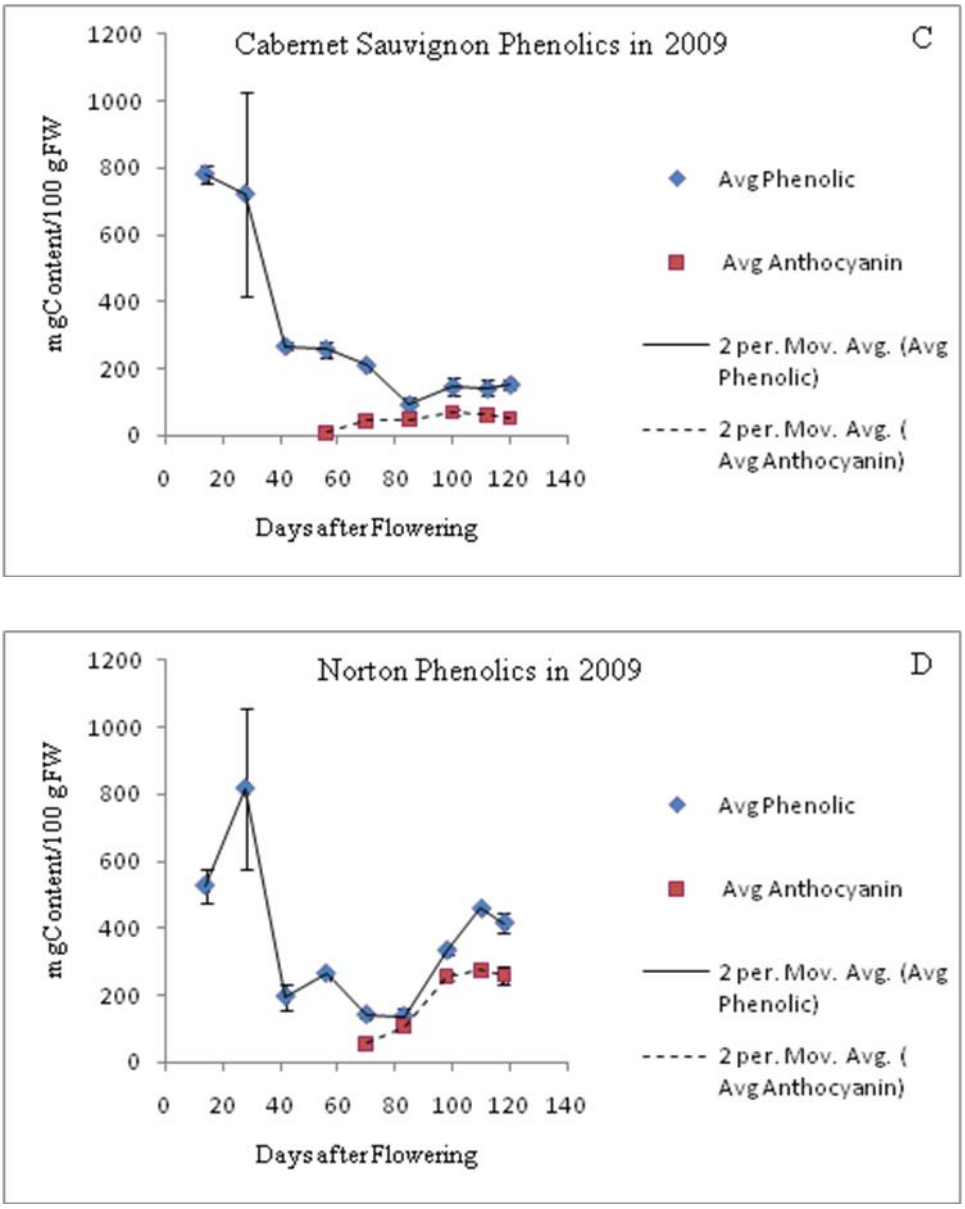
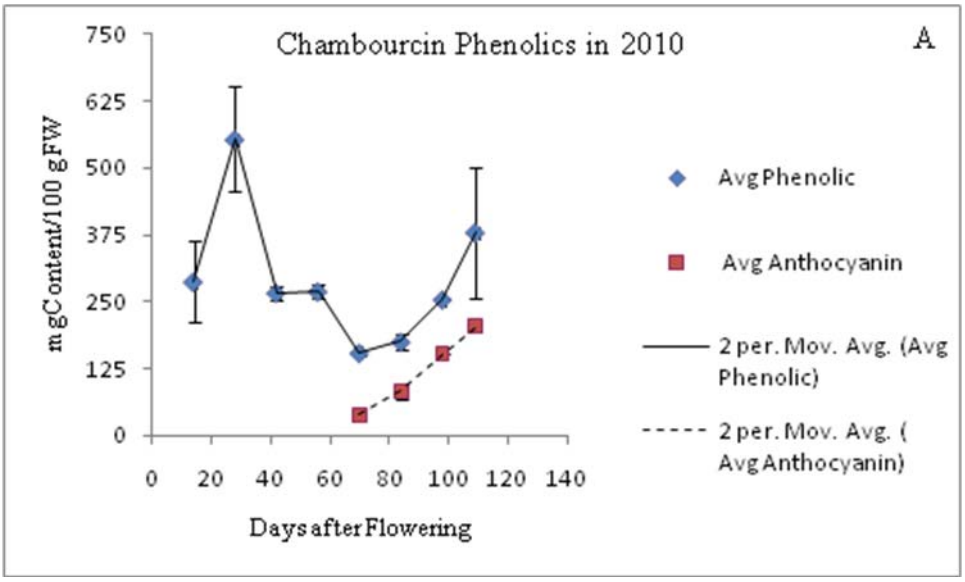


Figure 4.1. Phenolic content and anthocyanin content for the 2009 season of Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton from flowering on May 28th, June 1st, June 3rd, and June 5th, respectfully. All four cultivars were collected subsequently every two weeks until their harvest. For phenolic content and anthocyanin content, standard error bars (r=3) were included for every collection in which the phenolic compounds were detected in sufficient amounts (> 5 mg). Data points for Avg Phenolic and Avg Anthocyanin represent mean contents and bars with caps represent standard errors.


Chambourcin Phenolics in 2010
A
mg Content/100 g FW
750
625
500
375
250
125
0
0 20 40 60 80 100 120 140
Days after Flowering
Avg Phenolic
Avg Anthocyanin
2 per. Mov. Avg. (Avg Phenolic)
2 per. Mov. Avg. (Avg Anthocyanin)

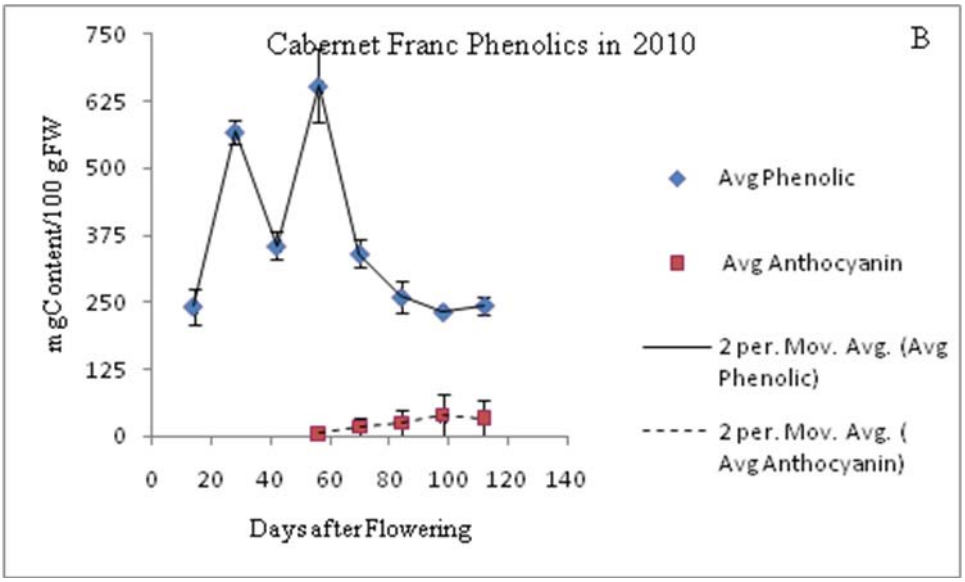

Cabernet Franc Phenolics in 2010
B
mg Content/100 g FW
750
625
500
375
250
125
0
0 20 40 60 80 100 120 140
Days after Flowering
Avg Phenolic
Avg Anthocyanin
2 per. Mov. Avg. (Avg Phenolic)
2 per. Mov. Avg. (Avg Anthocyanin)

Figure 4.2

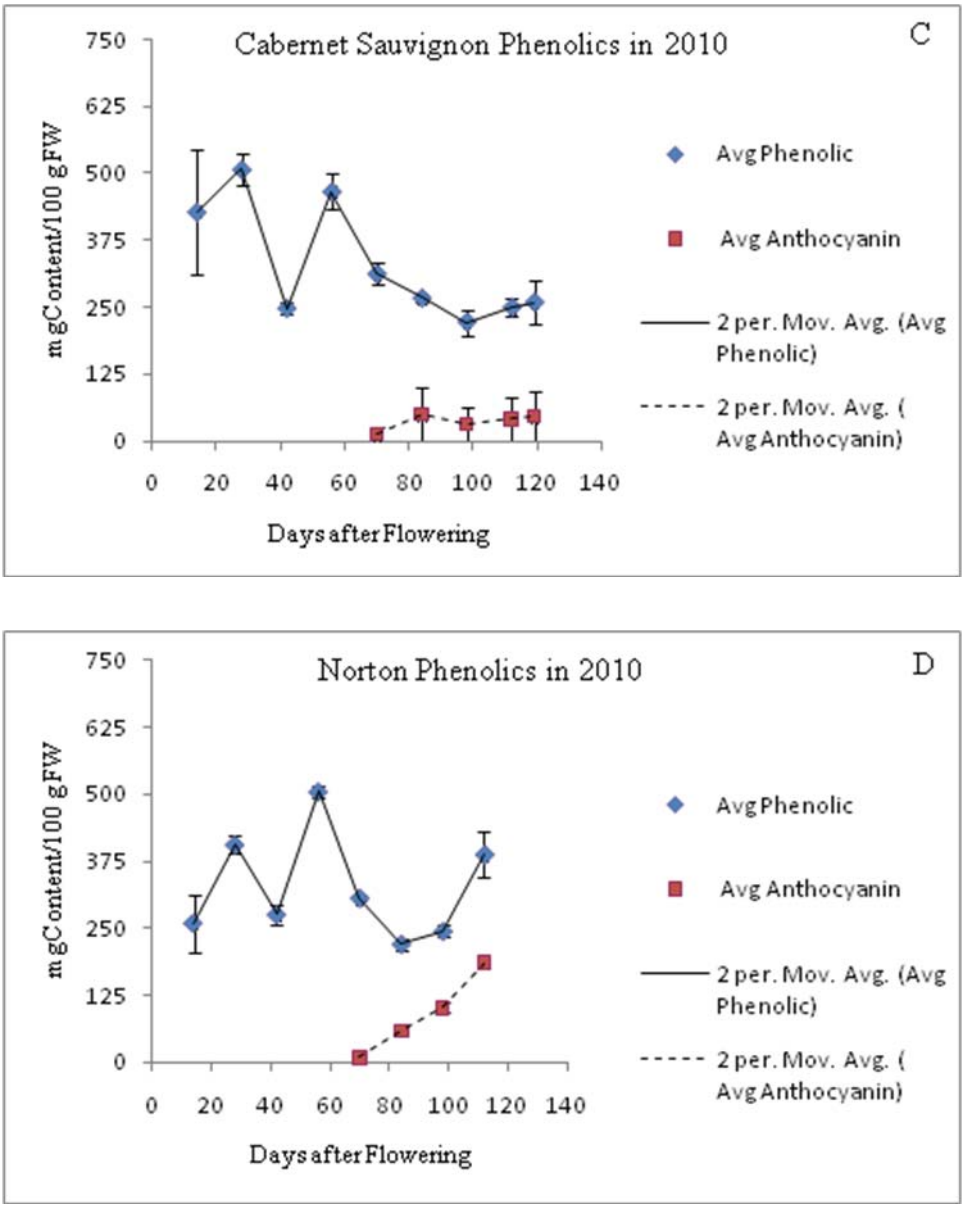


Figure 4.2. Phenolic content and anthocyanin content of Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton from flowering on June 3rd, 2010. All four cultivars were collected subsequently every two weeks until their harvest. For phenolic content and anthocyanin content, standard error bars (r=3) were included for every collection in which the phenolic compounds were detected in sufficient amounts (> 5 mg). Data points for Avg Phenolic and Avg Anthocyanin represent mean contents and bars with caps represent standard errors.

All cultivars also followed a pattern in comparison of their phenolic content and anthocyanin content at the 2009 harvest (Figure 4.1). Ranking of cultivars on the basis of highest to the lowest means at the harvest collection was the same for both the phenolic and the anthocyanin contents. Comparison of phenolic content to anthocyanin content at harvest was possible in the 2010 season (Figure 4.2). But unlike in the 2009 season, the ranking of the anthocyanin was not the same as the phenolics. While cultivar contents did not rank the same for the phenolic groups measured, the accumulation of phenolics was similar to pattern of accumulation of anthocyanins. A good representation of the coincidence of phenolic groups can be noted in Figure 4.2.

Change of berry mass later in the growing season was similar in both Cabernet Franc and Cabernet Sauvignon in 2009 (SEE APPENDICES; Appendix A.4 Wine Phenolics, Table A.4.1). A decline in the mean berry mass of Cabernet Franc was observed from the collection at 84 days after flowering through the collection at 113 days after flowering. For Cabernet Sauvignon, a decline of the mean berry mass happened from the collection 100 days after flowering through the collection 120 days after flowering. Changes in the mean berry mass later in the season were similar for the Chambourcin and Norton cultivars in 2009 (SEE APPENDICES; Appendix A.4 Wine Phenolics, Table A.4.1). From the collection's at 84 and 112 days after flowering for Chambourcin the berry mass remained quite stable. This was also the case in the Norton cultivar which had relatively stable means for berry mass at the collection 83 days after flowering through the collection 118 days after flowering.

Berry mass followed similar patterns of change of the means from 84 days (12 weeks) after flowering to the harvest collection in the 2010 season. The cultivars Cabernet Franc and Cabernet Sauvignon had slight decline from the collection at 12 weeks after flowering until the harvest collection at 16 weeks after flowering and 17 weeks after flowering, respectfully. The cultivar Chambourcin had decline in its mean mass as well, but unlike Cabernet Franc and Cabernet Sauvignon which saw some fluxuation with a slight increase for the collection at harvest compared to the collection preceding harvest, the mean Chambourcin berry mass continuously decreased from the collection at 12 weeks after flowering to the harvest collection at 16 weeks after flowering. The cultivar

Norton was the only cultivar with increase in mean berry mass from 12 weeks until the harvest collection. In fact, the increase was continuous in nature with slight increases in berry mass from collection to collection for the collection at 12 weeks after flowering until the harvest collection at 16 weeks after flowering.

Brix means changes were also similar among cultivars in 2009 (Table 4.2). Both Cabernet Franc and Cabernet Sauvignon saw non-significant decline of 0.4° Brix at harvest from the means of 18.2° Brix for Cabernet Franc and 17.5° Brix for Cabernet Sauvignon for the collection preceding the harvest collection. In Chambourcin and Norton cultivars, brix means were highest at harvest with a mean of 22.0° Brix for Chambourcin and 22.6° Brix for Norton which were non-significant increases of 0.2 and 0.6° Brix respectively for the two cultivars. It is also worth noting that the in the Cabernet Franc and Cabernet Sauvignon cultivars had means that were lower than the Chambourcin and Norton cultivar means at harvest.

Brix means patterns of change were similar in all cultivars in the 2010 grape growing season. From the collection at 12 weeks after flowering until the harvest collection there were only increases in the Brix means of the cultivars.

The pH change taking place later in grape maturity was also similar among cultivars in 2009 (Table 4.2). The pH change at the 99 days after flowering for Cabernet Franc and 100 days after flowering for Cabernet Sauvignon and when they were harvested at 122 and 120 days after flowering respectively was rather stable. For Cabernet Franc there was a -0.03 change in the pH means for the two previously mentioned collection times. For Cabernet Sauvignon, there was a 0.10 change in the pH means for the two previously mentioned times. The pH change was greater between the collection time of 98 days for both Chambourcin and Norton and their harvest at 126 and 118 days after flowering than what was noticed in Cabernet Franc and Cabernet Sauvignon. In the case of both Chambourcin and Norton the change in pH means was 0.23.

The pH change later in the grape maturity was similar among all cultivars in 2010. In all cultivars, noticeable change was found in mean pH from the collection at 12 weeks after flowering until the collection at 16 weeks after flowering. The cultivars Cabernet Franc

and Cabernet Sauvignon mean pH measurements increased by similar amounts. Cabernet Franc mean pH increased 0.16 pH, and Cabernet Sauvignon increased by 0.14 pH. Chambourcin and Norton mean pH values increase was approximately twice in magnitude as compared to Cabernet Franc and Cabernet Sauvignon with a mean increase of 0.30 pH for Chambourcin, and a mean increase of 0.29 pH for Norton.

Table 4.2. Cultivar phenolic sample berry mass, brix and pH measurements

collection days after flowering		berry mass[a] (g FW)		brix[a] (°Brix)		pH[a] (pH)	
2009	2010	2009	2010	2009	2010	2009	2010
				Cabernet Franc			
70	70	1.306 ± 0.033	1.608 ± 0.029	14.0 ± 0.5	14.5 ± 0.1	3.11 ± 0.08	3.19 ± 0.01
84	84	1.452 ± 0.024	1.847 ± 0.097	18.1 ± 0.5	17.2 ± 0.3	3.29 ± 0.05	3.25 ± 0.04
99	98	1.419 ± 0.095	1.670 ± 0.005	18.3 ± 0.6	21.4 ± 0.2	3.50 ± 0.07	3.38 ± 0.05
113	112	1.331 ± 0.068	1.776 ± 0.099	18.2 ± 1.4	21.6 ± 0.4	3.45 ± 0.05	3.41 ± 0.05
122		1.340 ± 0.164		17.8 ± 1.4		3.47 ± 0.08	
				Cabernet Sauvignon			
70	70	1.305 ± 0.112	1.398 ± 0.112	15.0 ± 0.5	11.9 ± 0.8	3.02 ± 0.01	3.02 ± 0.03
85	84	1.503 ± 0.043	1.351 ± 0.043	16.2 ± 0.9	14.8 ± 0.3	3.21 ± 0.01	3.09 ± 0.03
100	98	1.588 ± 0.088	1.461 ± 0.088	17.0 ± 0.6	16.7 ± 0.2	3.29 ± 0.04	3.20 ± 0.01
112	112	1.453 ± 0.126	1.400 ± 0.126	17.5 ± 0.9	19.7 ± 1.6	3.39 ± 0.01	3.12 ± 0.06
120	119	1.431 ± 0.039	1.437 ± 0.039	17.1 ± 0.5	21.7 ± 1.1	3.39 ± 0.04	3.23 ± 0.07
				Chambourcin			
71	70	1.969 ± 0.110	2.436 ± 0.064	12.1 ± 0.7	15.9 ± 1.0	2.82 ± 0.01	3.08 ± 0.05
84	84	2.198 ± 0.055	2.436 ± 0.064	17.4 ± 0.9	19.5 ± 0.4	2.98 ± 0.01	3.24 ± 0.02
98	98	2.150 ± 0.089	2.253 ± 0.055	19.1 ± 1.5	21.8 ± 0.6	3.15 ± 0.05	3.37 ± 0.03
112	109	2.198 ± 0.055	2.198 ± 0.055	21.8 ± 1.1	23.5 ± 0.8	3.27 ± 0.05	3.54 ± 0.01
126		2.587 ± 0.140		22.0 ± 0.6		3.38 ± 0.04	
				Norton			
70	70	1.100 ± 0.035	1.225 ± 0.074	18.6 ± 0.4	14.1 ± 0.4	2.91 ± 0.02	2.85 ± 0.02
83	84	1.271 ± 0.048	1.242 ± 0.073	19.5 ± 0.4	18.8 ± 0.6	3.04 ± 0.01	3.09 ± 0.03
98	98	1.190 ± 0.059	1.306 ± 0.033	22.5 ± 0.2	21.3 ± 0.4	3.15 ± 0.02	3.27 ± 0.01
110	112	1.201 ± 0.028	1.387 ± 0.041	22.0 ± 0.3	22.5 ± 0.4	3.31 ± 0.03	3.38 ± 0.02
118		1.233 ± 0.017		22.6 ± 0.2		3.38 ± 0.02	

[a] The mean value ± the standard error. Brix and pH measurements were begun at the collection at 70 (71 for Chambourcin in 2009) days after flowering and continued at all remaining collections of the particular season, the replicates were n=3.

4.3.2 Organic acid analysis

In analysis of variance for the entire collection of all four cultivars, the interaction between cultivar and temperature threshold was examined for significance ($p \leq 0.05$). Temperature threshold in this study is defined by the number of days up to the date of collection in which a minimum temperature of 20° C was either met or below this number of degrees Celsius for a table showing the temperature threshold days accumulated see Table 4.1. Testing the cultivar and year interaction for significance ($p \leq 0.05$) was also performed with the years being 2009 and 2010. For cultivar TA content, reported in Figure 4.3 and Figure 4.4, there was significance of the interaction of cultivar and temperature threshold with the cultivar ($p < 0.001$), temperature threshold ($p < 0.001$), and cultivar by temperature threshold ($p < 0.001$). Additionally the interaction of year on TA content was also significant ($p = 0.032$). The factor temperature threshold was not significant for MA content, also displayed in Figure 4.3 and Figure 4.4 beside respective TA contents for the cultivars ($p = 0.48$). Cultivar and year interactions revealed that the factors of cultivar ($p = 0.021$) and year ($p = 0.039$) were significant for MA content. When OA content, which can be viewed in Figure 4.3 and Figure 4.4, was analyzed for influential factors, it was found that cultivar, temperature threshold, and cultivar by temperature threshold were all highly significant with their p-values < 0.001. Year did not have significance on the OA content with a p-value of 0.124. The cultivar, temperature threshold, and cultivar by temperature threshold factors were all highly significant for CA content, displayed in Figure 4.3 and Figure 4.4 adjacent to respective OA contents, with p-values < 0.001. For CA content, year was also significant ($p = 0.003$). Investigation of the significance of cultivar and temperature threshold on berry mass of the entire collection revealed that the cultivar ($p < 0.001$), temperature threshold ($p < 0.001$), and cultivar by temperature threshold interaction ($p = 0.002$) were all found to be significant.

Table 4.3. Temperature thresholds for cultivar collection

collection (days after flowering)		temperature threshold[a]	
2009	2010	2009	2010
0	0	0	0
14	14	15	13
28	28	22	17
42	42	33	26
56	56	45	30
70	70	57	32
84	84	66	38
98	98	80	50
112	109* CH	94	60*
118* NT	112** CF & NT	102*	63**
120* CS	119* CS	102*	69*
122* CF		103*	
126* CH		108*	

[a] Number of days in which the mininum temperature was at or below 20° C. The number is representative of the total applicable days this threshold was attained leading up to the collection. * represents collection in which grapes were harvested. The cultivar abbreviation is included under collection for more clarity with the cultivar abbreviated as Cabernet Franc (CF), Cabernet Sauvignon (CS), Chambourcin (CH), Norton (NT). ** denotes that this is harvest collection for CF and NT. CS was collected but no harvested at that collection. Data was utilized from the University of Kentucky Agricultural Information Center to form number of temperature threshold days.

In determining whether the factors of temperature threshold and year were significant ($p \leq 0.05$) on the TA, MA, OA, and CA contents and berry mass of each cultivar, the following results were obtained; the accumulated days for temperature threshold of each cultivar can be found in Table 4.3. For Chambourcin the following were found for measurement of individual organic acids. TA, temperature threshold was highly

significant with a p-value < 0.001. For OA content (p < 0.001) and for CA content (p < 0.001), the temperature threshold was significant. Berry mass (p < 0.001) was significantly influenced by temperature threshold.

For the cultivar Cabernet Franc, the following was found for the factors of temperature threshold and year. For the TA content, temperature threshold was significant (p < 0.001) at the 0.05 level of significance. The OA content had highly significant influence due to the factor of temperature threshold (p < 0.001). The factors of temperature threshold (p = 0.019) and year (p = 0.019) were both significant for CA content in Cabernet Franc. In the case of the interaction with the factors on berry mass, temperature threshold was highly significant with a p-value < 0.001.

For the cultivar Cabernet Sauvignon the following results were found pertaining to the factors of temperature threshold and year. For TA content and for OA content, temperature threshold was highly significant with p-values < 0.001. For CA content, both temperature threshold (p < 0.001) and year (p = 0.011) were found to be significant and this also was the case with Cabernet Franc. For berry mass of Cabernet Sauvignon, temperature threshold was highly significant (p < 0.001).

For the cultivar Norton, the following results were found for the factors of temperature threshold and year. For TA content, temperature threshold (p = 0.008) and year (p = 0.006) were significant. For MA content, neither temperature threshold nor year was found to be significant as was the case with the other three cultivars that were also in the study. For OA content (p < 0.001) and CA content (p = 0.004), temperature threshold was a significant factor while year (p = 0.109, p = 0.434, respectfully) was not found to be significant. For berry mass of Norton, temperature threshold was highly significant with its p-value < 0.001.

Tartaric acid content per berry was highest either at harvest or the collection preceding harvest for cultivars except Norton which saw a decline in the mean tartaric acid content from its high at 10 weeks after flowering in 2009 (Figure 4.3). The tartaric acid content per berry was similar in its pattern of accumulation among cultivars in the 2010 season (Figure 4.4). The cultivars Cabernet Franc and Cabernet Sauvignon both had the highest

tartaric acid means at the harvest collection. At the harvest collection, Cabernet Franc had its highest value of 10.74 mg TA/berry, and Cabernet Sauvignon had its highest value of 11.86 mg TA/berry. For Chambourcin and Norton, high TA values were reached much earlier in the growing season.

Malic acid content had similarity in all cultivars with decline in MA content noticed after the 6 weeks after flowering collection for Cabernet Franc and after the 8 weeks after flowering collection for Cabernet Sauvignon, Chambourcin, and Norton in 2009 (Figure 4.3). For the 2010 growing season, malic acid content decline began prior to veraison in all cultivars except Cabernet Franc where it coincided with collection at the start of veraison (Figure 4.4). Decline began at 6 weeks after flowering in Chambourcin, and at 8 weeks after flowering for Cabernet Franc, Cabernet Sauvignon, and Norton.

Oxalic acid content followed similar patterns of accumulation for all cultivars in 2009 (Figure 4.3). From the collection at 0 weeks after flowering to the collection at 6 weeks after flowering there were increases in the OA content. Chambourcin showed an increase later in the growing season with its maximum mean OA content at the collection 16 weeks after flowering (Figure 4.3).

Oxalic acid content had similarities in the accumulation patterns among cultivars in 2010 (Figure 4.4). There were continuous increases in OA content in the early collections. Three of the cultivars had their highest mean OA content at the harvest collection. The only cultivar to not have its highest mean OA content at harvest was Norton which had its highest mean for OA/berry at 8 weeks after flowering.

Citric acid content had little pattern in berry content in the cultivars in the 2009 season (Figure 4.3). One of the only noticeable events was a decline in the mean content of CA from the collection at 10 weeks after flowering to the collection at 12 weeks after flowering for Cabernet Franc, Cabernet Sauvignon, and Norton cultivars. Accumulation of CA in general appeared to follow no particular pattern as there were fluctuations in mean CA content throughout the growing season. Nonetheless, the highs for CA content took place at harvest for the cultivars Cabernet Franc, Cabernet Sauvignon, and Chambourcin. For berry citric acid content in the 2010 grape growing season in Cabernet

Franc, Cabernet Sauvignon, and Chambourcin there was for the majority of collections a steady increase in mean CA content per berry lasting through 10 weeks after flowering (Figure 4.4). Cabernet Franc, Cabernet Sauvignon, and Chambourcin all either had the highest recorded citric acid content at the harvest collection or it equaled the high value of a previous collection. Cabernet Franc had its highest observed citric acid content at the week 10 collection.

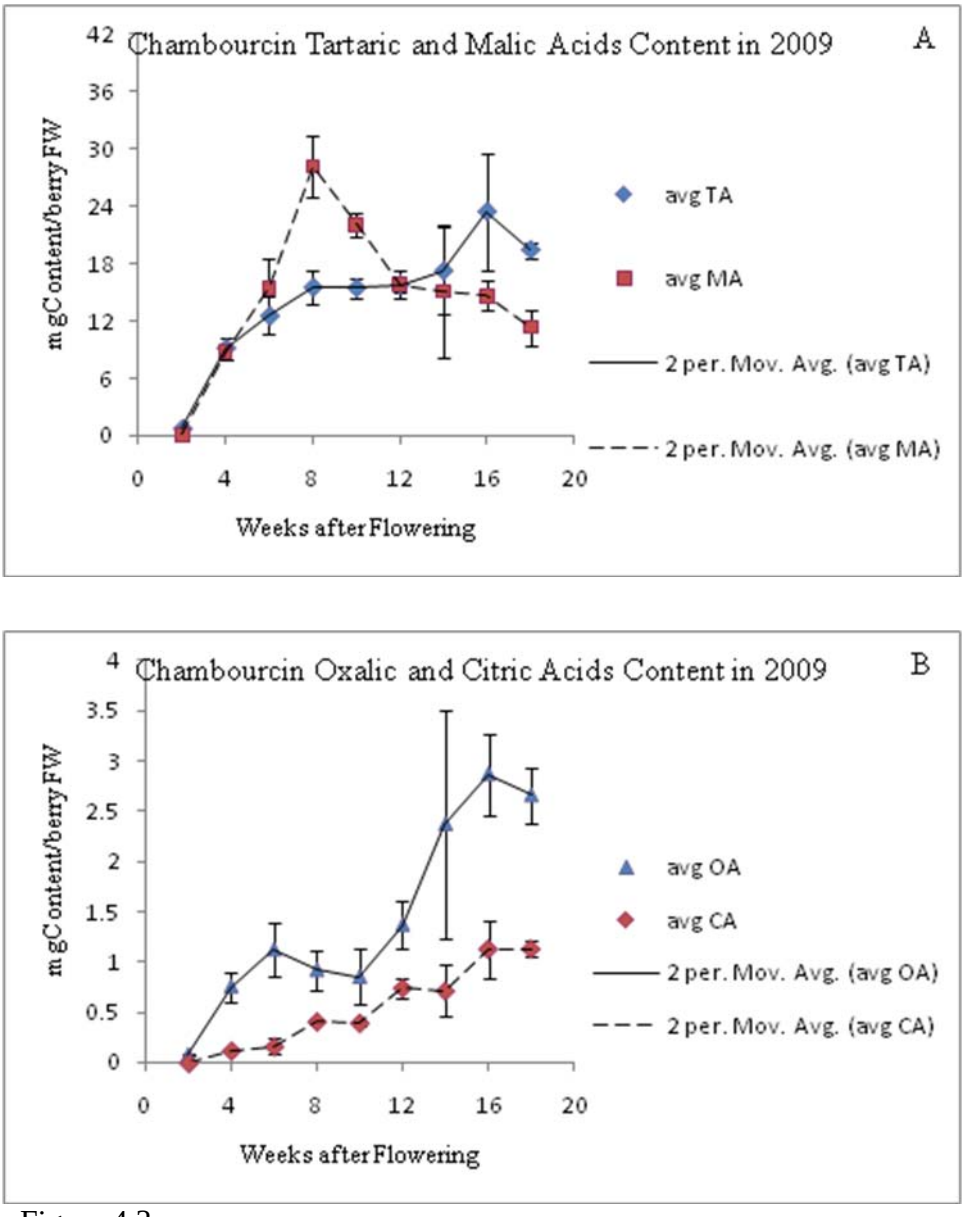
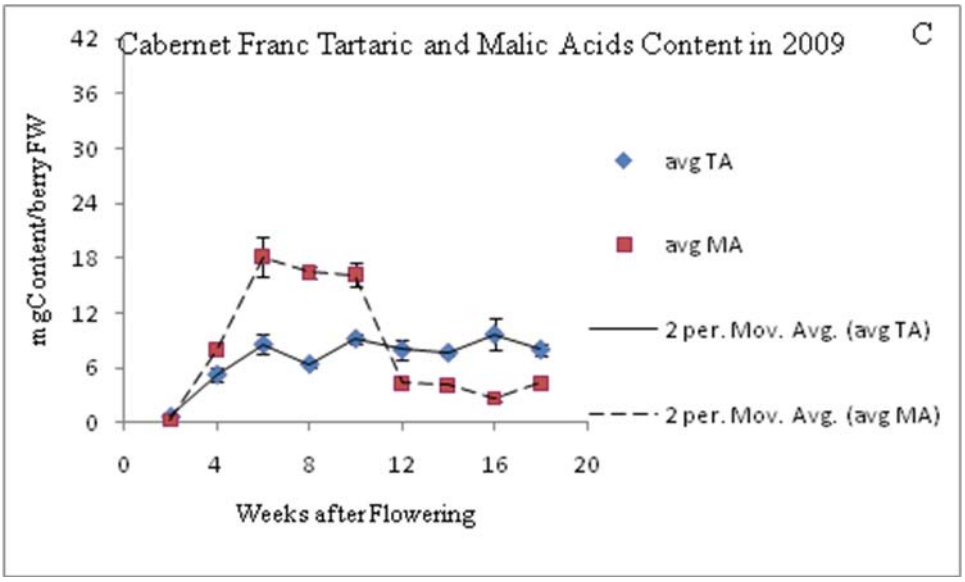


Figure 4.3


Cabernet Franc Tartaric and Malic Acids Content in 2009
C
mg Content/berry FW
Weeks after Flowering
avg TA
avg MA
2 per. Mov. Avg. (avg TA)
2 per. Mov. Avg. (avg MA)

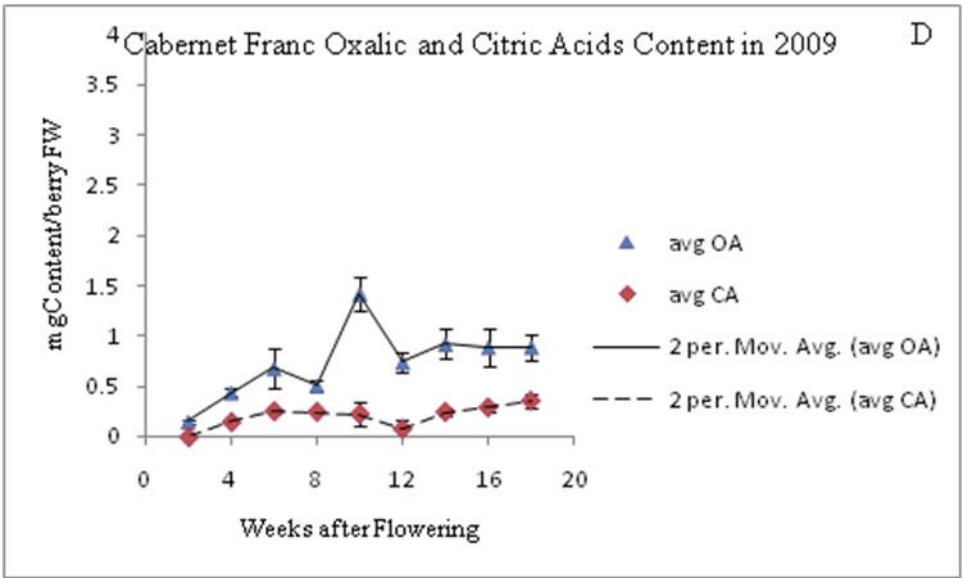
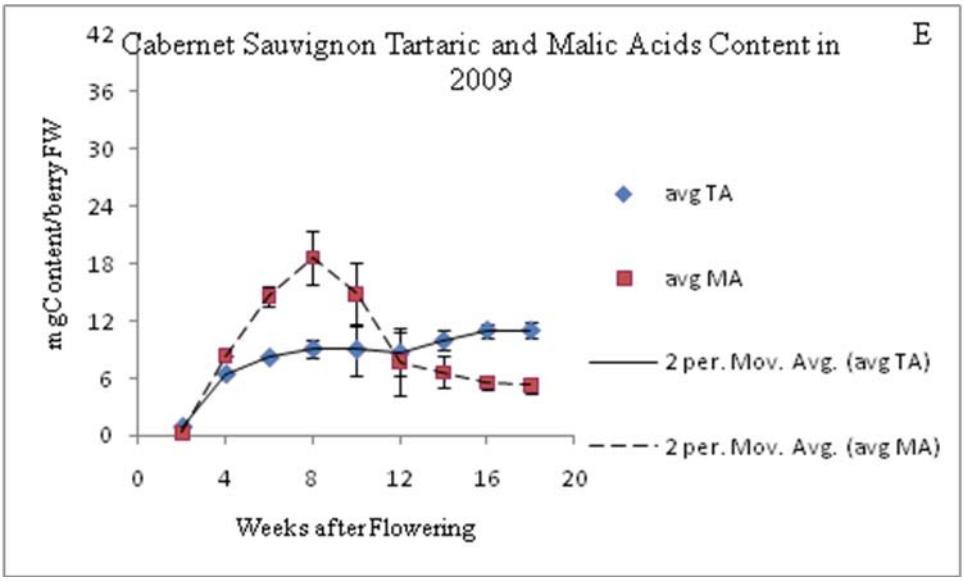

Cabernet Franc Oxalic and Citric Acids Content in 2009
D
mg Content/berry FW
Weeks after Flowering
avg OA
avg CA
2 per. Mov. Avg. (avg OA)
2 per. Mov. Avg. (avg CA)

Figure 4.3


Cabernet Sauvignon Tartaric and Malic Acids Content in 2009
E
mg Content/berry FW
Weeks after Flowering
avg TA
avg MA
2 per. Mov. Avg. (avg TA)
2 per. Mov. Avg. (avg MA)

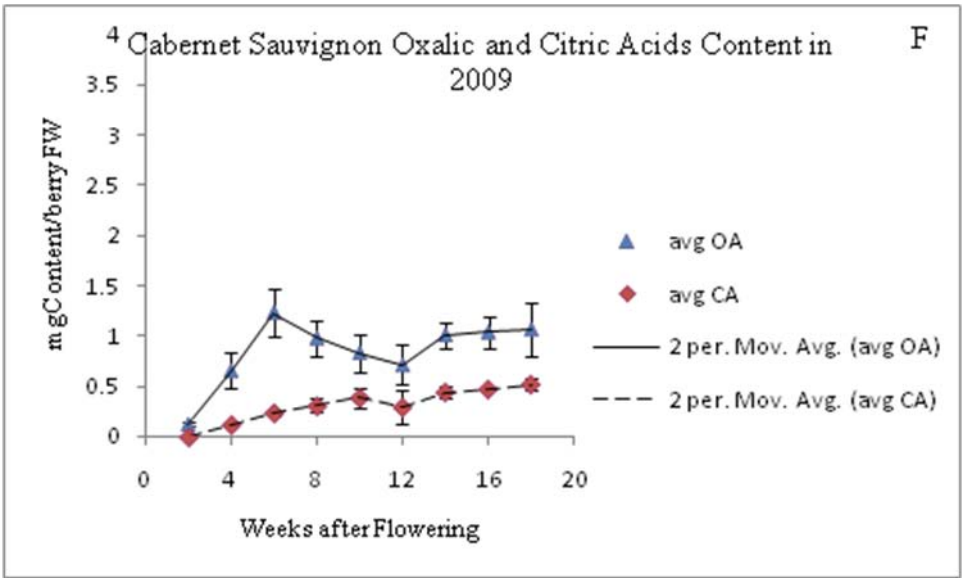
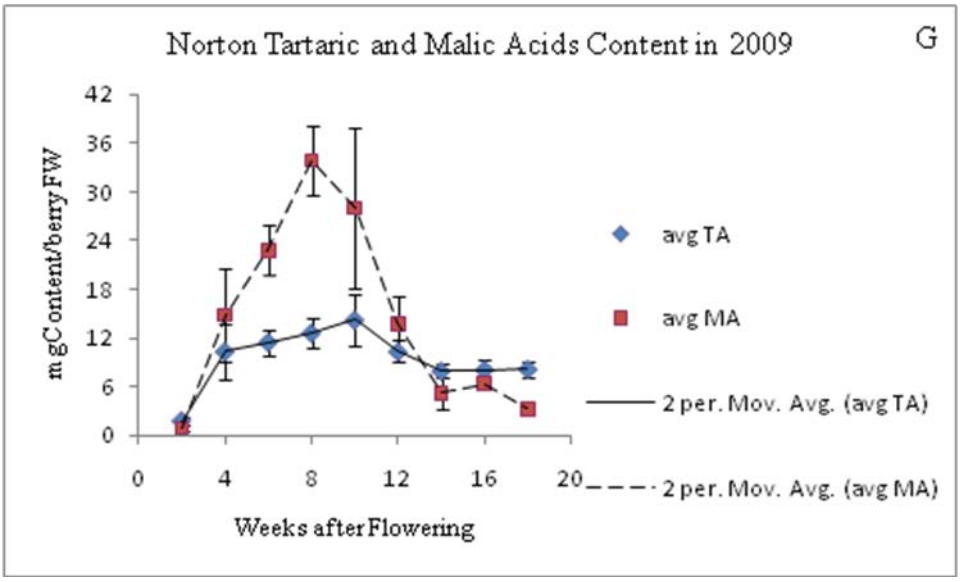

Cabernet Sauvignon Oxalic and Citric Acids Content in 2009
F
mg Content/berry FW
Weeks after Flowering
avg OA
avg CA
2 per. Mov. Avg. (avg OA)
2 per. Mov. Avg. (avg CA)

Figure 4.3



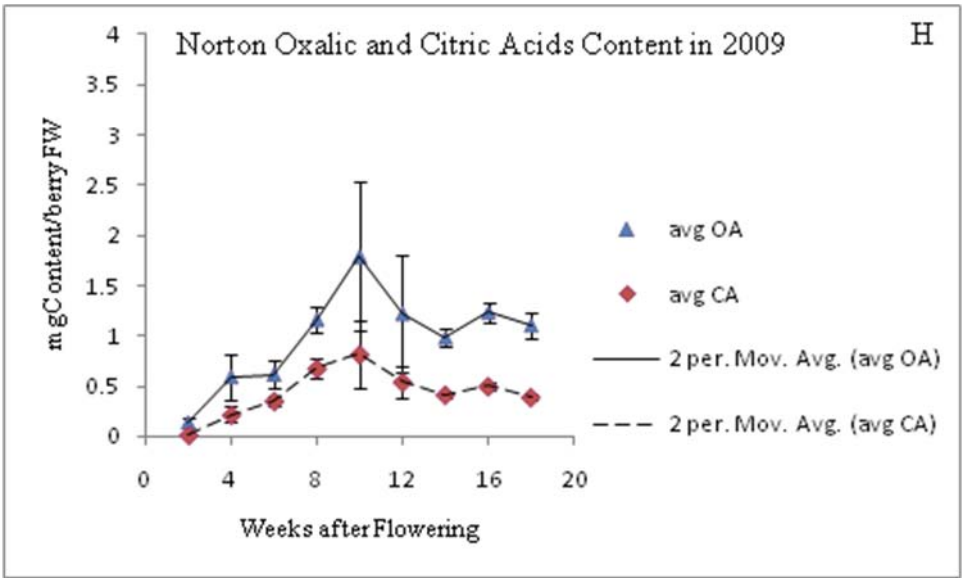


Figure 4.3. Tartaric acid and malic acid content (**A, C, E, G**) for the grape cultivars Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton, respectfully and oxalic acid and citric acid content of Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton, (**B, D, F, H**). The collecting starting at two weeks after flowering on June 10th, June 15th, June 17th, and June 19th, 2009, respectfully, and all four cultivars were collected subsequently every two weeks until their harvest. Data points for Avg TA and MA, or OA and CA, represent mean contents and bars with caps represent standard errors.

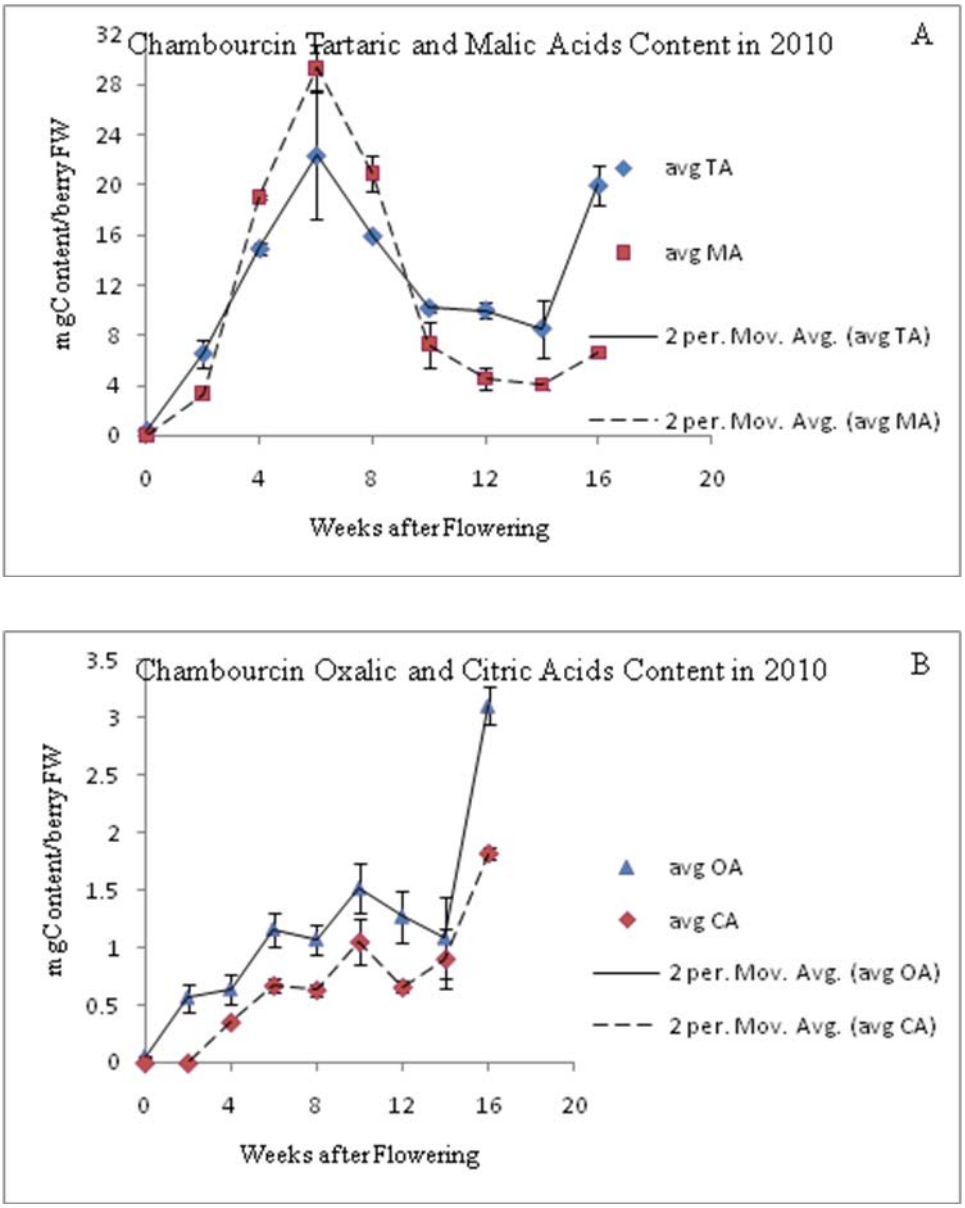
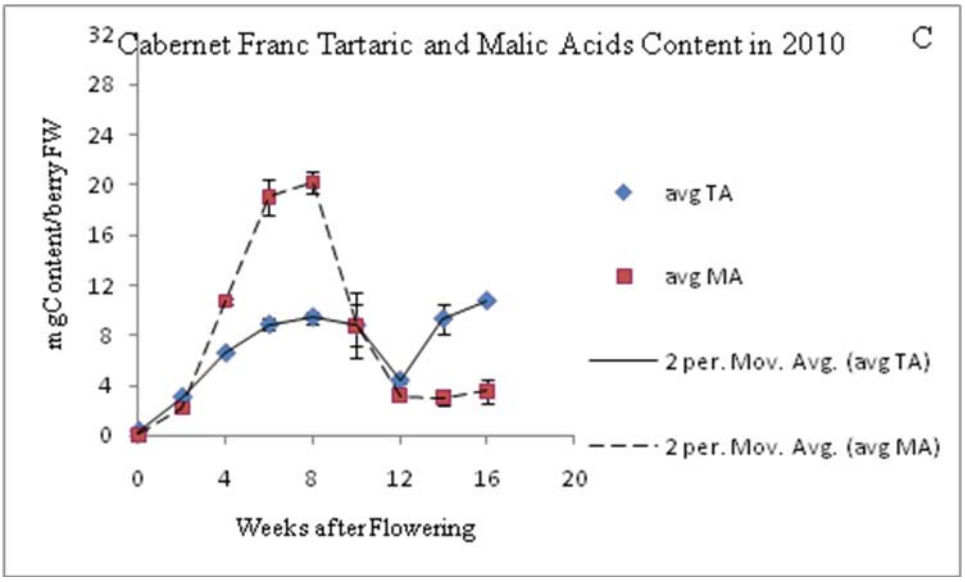

Chambourcin Tartaric and Malic Acids Content in 2010
A
mg Content/berry FW
Weeks after Flowering
avg TA
avg MA
2 per. Mov. Avg. (avg TA)
2 per. Mov. Avg. (avg MA)
Chambourcin Oxalic and Citric Acids Content in 2010
B
mg Content/berry FW
Weeks after Flowering
avg OA
avg CA
2 per. Mov. Avg. (avg OA)
2 per. Mov. Avg. (avg CA)

Figure 4.4


Cabernet Franc Tartaric and Malic Acids Content in 2010
C
mg Content/berry FW
Weeks after Flowering
avg TA
avg MA
2 per. Mov. Avg. (avg TA)
2 per. Mov. Avg. (avg MA)

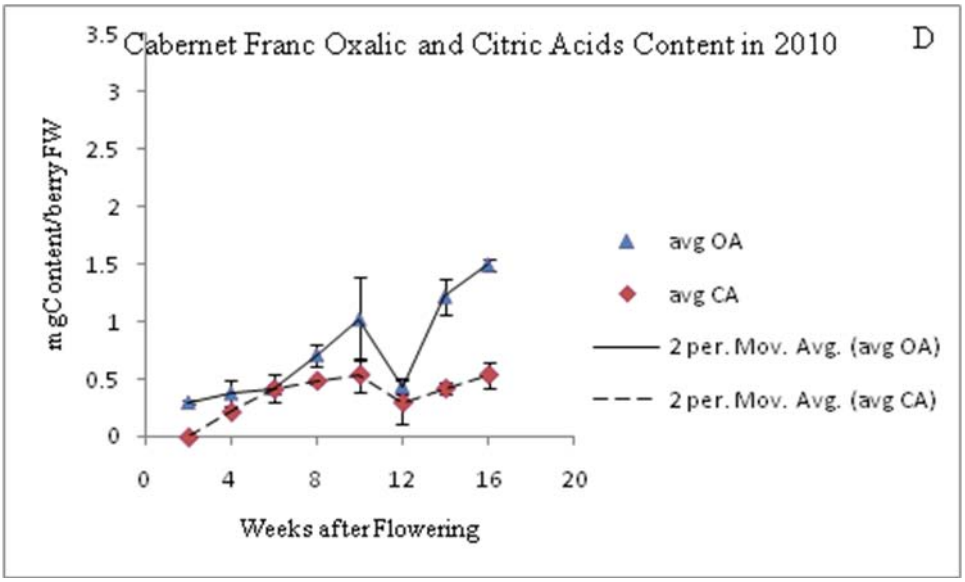
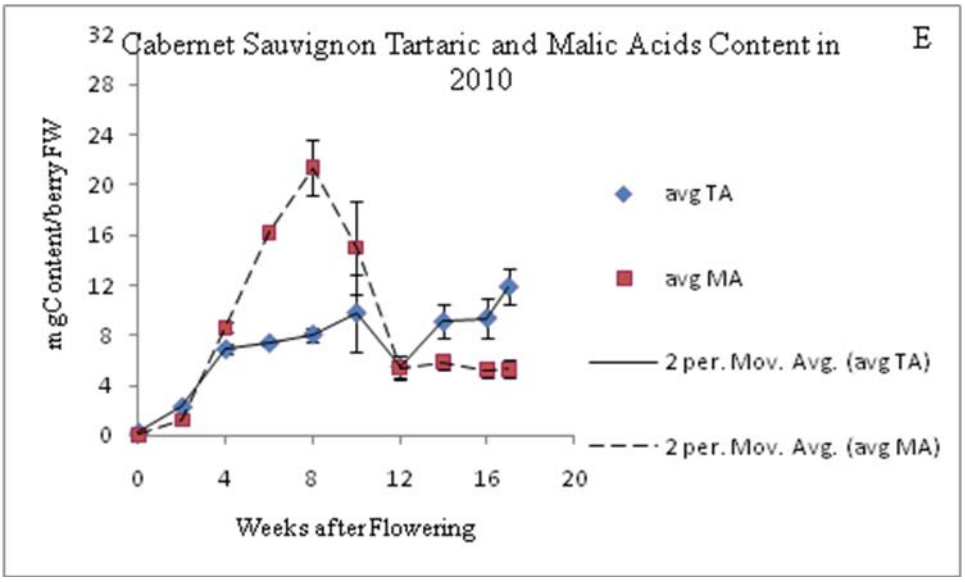

Cabernet Franc Oxalic and Citric Acids Content in 2010
D
mg Content/berry FW
Weeks after Flowering
avg OA
avg CA
2 per. Mov. Avg. (avg OA)
2 per. Mov. Avg. (avg CA)

Figure 4.4


Cabernet Sauvignon Tartaric and Malic Acids Content in 2010
E
mg Content/berry FW
Weeks after Flowering
avg TA
avg MA
2 per. Mov. Avg. (avg TA)
2 per. Mov. Avg. (avg MA)

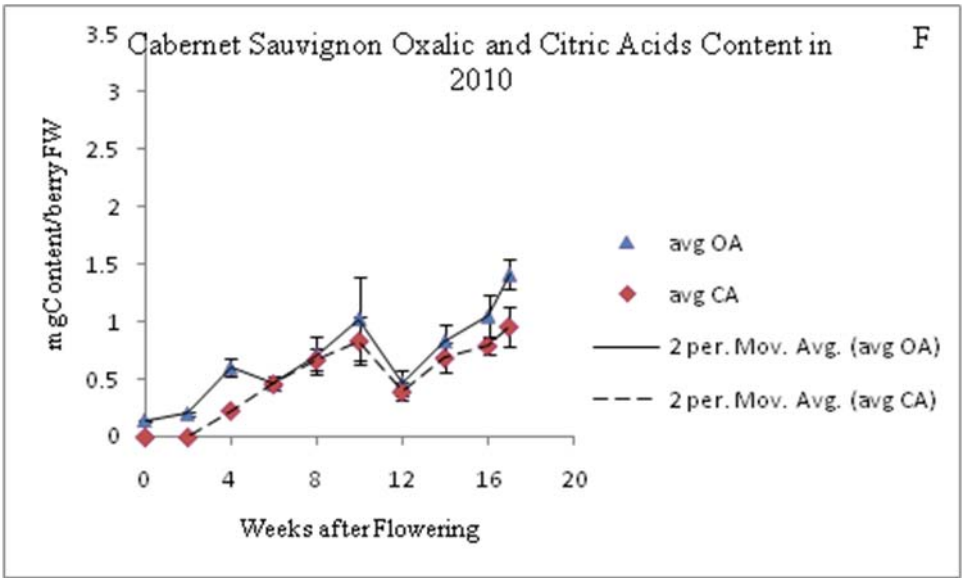
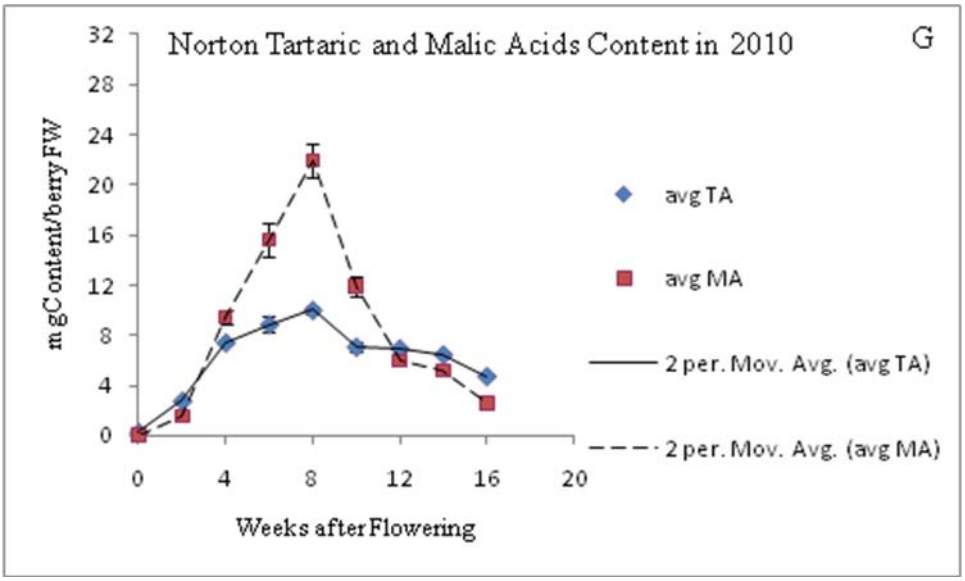

Cabernet Sauvignon Oxalic and Citric Acids Content in 2010
F
mg Content/berry FW
Weeks after Flowering
avg OA
avg CA
2 per. Mov. Avg. (avg OA)
2 per. Mov. Avg. (avg CA)

Figure 4.4



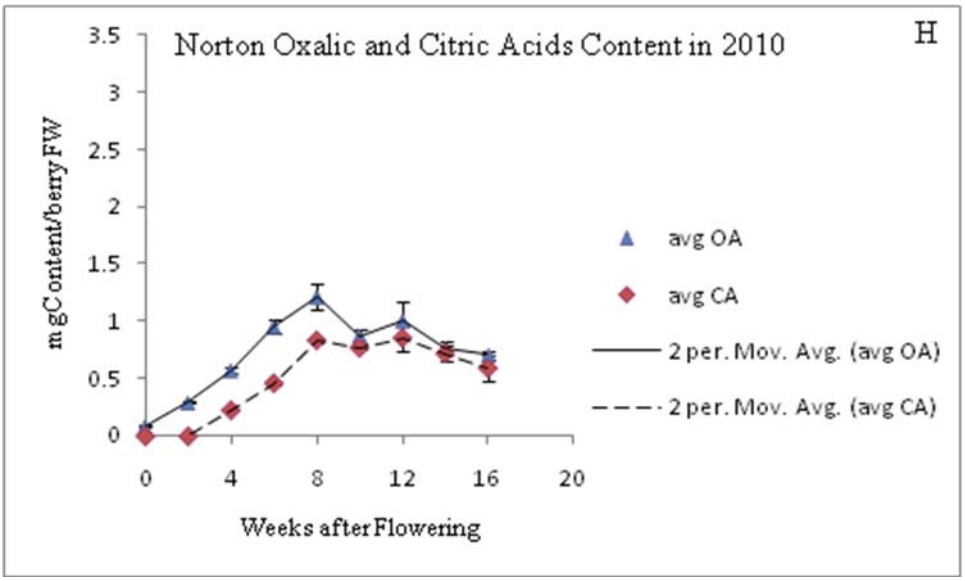


Figure 4.4. Tartaric acid and malic acid content (**A, C, E, G**) for the grape cultivars Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton, respectfully and oxalic acid and citric acid content of Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton, (**B, D, F, H**). The collecting starting at flowering on June 3rd, 2010, and all four cultivars were collected subsequently every two weeks until their harvest. Data points for Avg TA and MA, or OA and CA, represent mean contents and bars with caps represent standard errors.

Titratable acidity was investigated for potential connections that it has with the organic acid contents for the cultivars (Table 4.4). In all cultivars examined it was at its highest value for mean titratable acidity, expressed as g titratable acids per liter of juice, at the first collection in which it was measured which was 10 weeks after flowering. In 2010, titratable acidity saw a decline in all cultivars after the first time it was measured at 10 weeks after flowering (Table 4.4). Cabernet Franc, Chambourcin, and Norton all saw continuous decline from the initial measurement until the harvest collection for the three at 16 weeks after flowering.

Table 4.4. Cultivar organic acid sample titratable acidity, brix, and pH measurements

collection weeks after flowering		titratable acidity[a] (g titratable acids/ L juice)		brix[a] (°Brix)		pH[a] (pH)	
2009	2010	2009	2010	2009	2010	2009	2010
				Cabernet Franc			
10	10	12.35 ± 0.24	19.17 ± 0.55	14.0 ± 0.5	14.5 ± 0.1	3.11 ± 0.08	3.19 ± 0.01
12	12	5.25 ± 0.60	11.73 ± 0.67	18.1 ± 0.5	17.2 ± 0.3	3.29 ± 0.05	3.25 ± 0.04
14	14	6.97 ± 0.60	11.33 ± 0.69	18.3 ± 0.6	21.4 ± 0.2	3.50 ± 0.07	3.38 ± 0.05
16	16	8.28 ± 0.87	9.73 ± 0.67	18.2 ± 1.4	21.6 ± 0.4	3.45 ± 0.05	3.41 ± 0.05
18		7.70 ± 0.28		17.8 ± 1.4		3.47 ± 0.08	
				Cabernet Sauvignon			
10	10	21.94 ± 0.99	23.50 ± 0.66	15.0 ± 0.5	11.9 ± 0.8	3.02 ± 0.01	3.02 ± 0.03
12	12	13.55 ± 0.26	16.13 ± 0.94	16.2 ± 0.9	14.8 ± 0.3	3.21 ± 0.01	3.09 ± 0.03
14	14	10.84 ± 0.08	11.90 ± 0.18	17.0 ± 0.6	16.7 ± 0.2	3.29 ± 0.04	3.20 ± 0.01
16	16	7.99 ± 1.14	12.80 ± 0.26	17.5 ± 0.9	19.7 ± 1.6	3.39 ± 0.01	3.12 ± 0.06
18	17	10.20 ± 0.75	9.05 ± 0.79	17.1 ± 0.5	21.7 ± 1.1	3.39 ± 0.04	3.23 ± 0.07
				Chambourcin			
10	10	21.38 ± 0.78	13.03 ± 1.04	12.1 ± 0.7	15.9 ± 1.0	2.82 ± 0.01	3.08 ± 0.05
12	12	12.13 ± 1.31	9.53 ± 0.38	17.4 ± 0.9	19.5 ± 0.4	2.98 ± 0.01	3.24 ± 0.02
14	14	9.86 ± 0.28	8.64 ± 0.24	19.1 ± 1.5	21.8 ± 0.6	3.15 ± 0.05	3.37 ± 0.03
16	16	8.88 ± 0.17	8.51 ± 0.16	21.8 ± 1.1	23.5 ± 0.8	3.27 ± 0.05	3.54 ± 0.01
18		8.09 ± 0.28		22.0 ± 0.6		3.38 ± 0.04	
				Norton			
10	10	29.81 ± 2.27	28.31 ± 0.54	18.6 ± 0.4	14.1 ± 0.4	2.91 ± 0.02	2.85 ± 0.02
12	12	16.88 ± 0.19	16.33 ± 0.18	19.5 ± 0.4	18.8 ± 0.6	3.04 ± 0.01	3.09 ± 0.03
14	14	11.56 ± 0.23	11.08 ± 0.11	22.5 ± 0.2	21.3 ± 0.4	3.15 ± 0.02	3.27 ± 0.01
16	16	9.14 ± 0.43	8.58 ± 0.41	22.0 ± 0.3	22.5 ± 0.4	3.31 ± 0.03	3.38 ± 0.02
18		9.80 ± 0.22		22.6 ± 0.2		3.38 ± 0.02	

Table 4.4 (Continued). Cultivar organic acid sample titratable acidity, brix, and pH measurements

[a] The mean value ± the standard error. Titratable acidity, brix, and pH measurements were begun at the collection at 10 weeks after flowering and continued at all remaining collections of the particular season, the replicates were n=3.

Berry mass was recorded for the organic acid analysis in 2009 and the following season in 2010 (SEE APPENDICES; Appendix A.4 Wine Phenolics, Table A.4.3). Cultivars were similar in their changes of mass. From the collection 0 days after flowering through 10 weeks after flowering there were the most substantial increases in the mean berry mass in all cultivars. This was followed by a lag phase in berry growth for the 12 week after flowering collection. Norton did not have a decline for the 12 week after flowering collection. Chambourcin saw an increase in mean berry mass at 12 weeks after flowering but then had a decline in the mean berry mass at the following 14 weeks after flowering collection. All of the cultivars except Norton had an increase in mean berry mass from the collection preceding harvest at 16 weeks after flowering to the collection at harvest 18 weeks after flowering (SEE APPENDICES; Appendix A.4 Wine Phenolics, Table A.4.3). In 2010, berry mass recorded for the organic acid analysis was found to increase greatest from the collection at 0 weeks after flowering until the collection at 10 weeks after flowering for Cabernet Franc, Chambourcin, and Norton and Cabernet Sauvignon had greatest increase in mean berry mass until 12 weeks after flowering (SEE APPENDICES; Appendix A.4 Wine Phenolics, Table A.4.3). This contrasts with the 2009 grape growing season in which a decrease was found during the lag phase for both Cabernet Franc and Cabernet Sauvignon. Unlike in the 2009 season in which three cultivars had increases in berry mass from the collection before harvest to the harvest collection, there was only one cultivar that exhibited an increase in mean berry mass from the collection preceding harvest to the harvest collection in 2010; that cultivar was Cabernet Sauvignon which did take longer to reach veraison and correspondingly the lag phase of growth (SEE APPENDICES; Appendix A.4 Wine Phenolics, Table A.4.3).

The pH and brix readings were done only once for each replicate, so they are the same as mentioned for the Results, Phenolics analysis 4.3.1, and are represented in Table 4.2 and

also in Table 4.4. As a reminder, the cultivars Cabernet Franc and Cabernet Sauvignon had similar trends in both the pH means and the brix means during the growing season of 2009. The same was true for the cultivars Chambourcin and Norton which were similar in their pattern of change for both the pH means and the brix means throughout the growing season in 2009. In 2010, all cultivars had increases in their brix and pH measurements from the initial measurements at 10 weeks after flowering until their harvest collection. For Cabernet Franc, Chambourcin, and Norton the increase in the brix and pH measurements was continuous in nature. Cabernet Sauvignon had continuous increase in brix means throughout the 2010 season.

4.4 Discussion

4.4.1 Phenolics

The decline in phenolics for the collection at flowering until the collection at ten weeks post-flowering is explained by the nature of the grape berry. A substantially greater surface area to volume ratio in immature berries as compared to berries that have matured would mean that the fresh weight phenolic content for immature berries should be higher than the fresh weight phenolic content of mature grapes. In Singleton (1966), the author's explanation was stating that there was decline in the surface area to volume ratio of the berry by using the terminology of dilution of phenolics. Phenolic content was found in general to be similar per given area of berry skin (Singleton, 1966). Phenolic dilution is due to increased berry weight which takes place due to the berry volume increase that happens through berry cell division first, followed by enlargement of the berry cells.

Another underlying plant physiology principal which can be accurately measured during berry development is the osmotic potential difference between the berry and plant which changes with the major changes of berry development. The increase in berry mass and volume is due to an increase in berry water content to adjust for an increasingly more negative osmotic potential (Smart and Coombe, 1983). In our study, there was decline of

phenolic content from the content found at veraison (Figure(s) 4.1 & 4.2). Similar results were found in Giovanelli and Brenna (2007) where a decline in veraison phenolic content from the time of veraison onward was the case for three cultivars in the study.

Rainfall during grape development can reduce sugar content of the grape juice likely by mechanisms such as increased berry size resulting from excessive water supply. What may have accounted for slower accumulation of brix in the 2009 season might have been rainfall that had occurred post-veraison which did not cause decreases in soluble solid content, but did cause for slow increase in ° Brix to reached desired levels for harvest maturity. An accelerated rate of brix increase was found in the following season in 2010 which had lower rainfall accumulation from post-veraison to harvest. Harvest time in 2010 was earlier than in 2009, see Table 4.5 for rainfall accumulation in 2009 and 2010.

Interestingly, the berry size was not found to be higher in 2009 which saw an increased rainfall during the growing season compared to the 2010 growing season (SEE APPENDICES; Appendix A.4 Wine Phenolics, Table A.4.1). Some regions, such as the Mediterranean, and in the U.S., Napa Valley in California, rely heavily on rainfall primarily accumulated during the winter months which is capable of storage in the vineyard soils. A potential explanation to this rather complex association of climatic factors, such as rainfall, with the berry size during grape development is that berry size could be profoundly influenced by water-storage capability of soil and therefore early-season rainfall (Smart and Coombe, 1983). In 2010, rainfall in the early season was nearly equal to early-rainfall in 2009 (Table 4.5). Early grape development is characterized by a period of rapid cell devision prior to cell-expansion later in the season. Often, fruit size has been known to be determined by initial cell-division rather than cell-elongation, as noted in a review by Dokoozlian (2000), which could account for the greater berry mass in 2010 than in the 2009 season, although the 2009 season did have more total accumulated rainfall, see Table 4.5 for rainfall accumulation in 2009 and 2010. In grapes, this result was found by Smart and Bingham (1974), where the observation that water-stressed grapes earlier in the grape season which also possessed slower rate of cell-division than well-watered grapes produced grapes with low mass at grape maturity compared with the properly watered grapes serving as the control. Regarding

precipitation, excessive precipitation can delay the ripening process in grapes, particularly in Stages I and II (Jackson and Lombard, 1993). A concise review on berry development highlights the major stages of development, with the first two stages characterized by rapid cell division in stage 1 and lag phase in stage 2 (Dokoozlian, 2000).

Table 4.5 Rainfall accumulation in 2009 and 2010

month (December to September) 2008-2009 and 2009-2010	rainfall total[a] (inches of precipitation) 2008-2009	2009-2010
December	6.03	4.03
January	10.35	7.23
February	12.88	8.84
March	15.27	10.06
April	20.06	12.35
May	26.1	22.3
June	31.29	26.89
July	38.86	32.95
August	43.39	33.75
September	49.29	34.36

[a] The number represents inches of rainfall accumulated during the season through the end of the month listed in the row. Data obtained from University of Kentucky Agricultural Weather Center <http://wwwagwx.ca.uky.edu/>.

In regard to "phenolic priming" affect that was mentioned in the Results, Phenolics analysis 4.3.1, there have been previous studies in which this was observed. In Giovanelli and Brenna (2007), an increase in total phenolics occurred at the time corresponding to veraison followed by a decline post-veraison. While the increase in anthocyanin content does begin at veraison, the increase in anthocyanins alone does not account for the total increase in phenolics. This is why a description of the increase of total phenolics at veraison should be termed more of a priming of the phenylpropanoid pathway. This is due to the fact that phenolic levels do increase at the start of veraison,

but there is low content of anthocyanins at this time, however there was a moderate change in the phenolic content of the grapes. The effect was also observed in Singleton (1966) where the phenolics would have a moderate increase later in July or in early August which would correspond to the time before coloration or right as anthocyanin accumulation in the skin of the grapes began. This finding was also supported by this study in which the majority of cultivars displayed the highest phenolic content at the collection at 56 days after flowering, or 8 weeks from the observation of full flowering. In Giovanelli and Brenna (2007), the highest phenolic content's of the three cultivars included in their study was reached before veraison.

Regarding the anthocyanin accumulated by the cultivars at harvest, cultivar performance defined by ability to achieve full and high coloration within a region certainly had a role in berry coloration (Kliewer and Torres, 1972). In both the 2009 and 2010 growing seasons, the cultivars Chambourcin and Norton accumulated high levels of anthocyanins, regarding the mean content of milligrams of malvidin-3-glucoside equivalents per 100 gram of fresh-weight berries, compared to the two *vinifera* cultivars Cabernet Franc and Cabernet Sauvignon , see Figure 4.1 for 2009 and Figure 4.2 for 2010. A very striking increase post-veraison to harvest can be observed on the graphs of Chambourcin and Norton, while Cabernet Franc and Cabernet Sauvignon experienced minimal change in their anthocyanin content post-veraison. A reasonable conclusion to this observation from the data of both seasons is that cultivar performance does have a role in anthocyanin accumulation determined for the four cultivars. Chambourcin and Norton are better-suited for achieving high coloration in the climate of Kentucky and regions displaying similar environments than the cultivars Cabernet Franc and Cabernet Sauvignon. Such an opinion would be held in a previous study done in Australia in which four cultivars were grown in dramatically different climates and the total anthocyanin accumulation was found to be best in the warmer climates of the Padthaway and Riverland growing regions which allowed for optimal coloration for the four red wine cultivars which must have not been cool-season grapes, as seemingly the cultivars did not have reduction in their anthocyanin accumulation in higher temperatures, but rather these cultivars achieved greater accumulation in warmer climates (Cozzolino *et al.*, 2010).

Although not determined in this study, hours of light interception was found by Vilanova *et al.* (2009) to help grapes attain full coloration; and this could be an explanation as to why warmer climates allow certain cultivars to achieve better coloration due to more light-hour accumulation during and after the onset of veraison. Such an opinion is supported in the study by Cozzolino *et al.* (2010) which included four cultivars in which the two regions in Australia attaining the highest average anthocyanin accumulation were warmer regions. In this study, the cultivar Chambourcin had a higher mean anthocyanin content in 2010 in which there were fewer days that were below the temperature of 20° C, which although under-substantiated does suggests cultivar performance of Chambourcin does improve in warmer climates versus climates considered cooler climates.

The phenolic content, as well as anthocyanin content, in the 2009 and 2010 seasons was overall similar for each cultivar with no substantial changes taking place in phenolic content or anthocyanin content from the first and second seasons. Such an observation was mentioned in the Results, Phenolics analysis 4.3.1, when phenolic and anthocyanin contents of the four cultivars were ranked from highest mean to lowest mean and the orders of the cultivars had at the most one change of rank, either higher or lower, from the 2009 season to the 2010 season which suggests that phenolic composition of cultivars is based more on the cultivar ability as based on the cultivar's genetics versus being altered by the environment. This further supports the theory of cultivar performance, which is coloration that is reached in the climate where the cultivar has been established.

Red wine grape cultivars vary much less than white wine grapes from year to year in their phenolic composition according to the study of Vilanova *et al.* (2009), which did find that light-hours improved coloration, but the underlying contribution of the cultivar on phenolic composition still exists. The underlying genetic contribution is what dictates the cultivar's potential for high coloration, and the range of total phenolic content of the cultivar. Support for a cultivar's genetic contribution to phenolic composition is also supported by the study of McCallum *et al.* (2009) where a lack of correlation existed between the widely used measurement of degrees Brix to determine grape maturity and the anthocyanin content. The lack of correlation of an indicator of berry maturity and the

anthocyanin content would allow for the argument of cultivar genetic contribution to the phenolic composition of berries to be plausible. With season-to-season variation there seems to exist a confined range of the phenolic composition of a cultivar. This is evident in graphs of the cultivars that even though phenolic content and anthocyanin contents of the four cultivars were different in the 2009 season compared to the 2010 season, the respective trendlines of each cultivar are remarkably similar for both seasons (Figure 4.1 & Figure 4.2). The similarity in trendlines from the first to the second season does indicate genetic contribution to the phenolic composition of the berries of a cultivar. The question of whether the berry weight is a good determinant of anthocyanin content of the berries is still yet to be determined. Our study observed that simply stating that surface area to volume ratio is what determines the concentration of anthocyanin per mass of berries is not true which is the opposite of what was found in the study by Esteban *et al.* (2001). The heaviest grapes were from Chambourcin and the lighter grapes from Norton (Table 4.2). Even though the berries of these two cultivars have greatly different masses, the anthocyanin content of 100 g FW of berries of these two cultivars was similar in both seasons of our study (Figure 4.1).

Regarding the total phenolic content, support for increasing berry mass and declining phenolic concentration was found in our study as shown in Table 4.2 and in the cultivar graphs of Figures 4.1 and 4.2. The observed increase in phenolic concentration as berries approach the harvest collection is due to the small change in mass of the berries which is typically 100 or 200 mg, but for berries this represents a significant reduction of the berry mass and would support the claim that the greater berry mass reduces the phenolic concentration. The appropriate term dilution was used to describe reduced phenolic concentration of grape berries as berry weight increased (Singleton, 1966). This is a logical conclusion on phenolic concentration of grape berries due to the fact that as the berry becomes a sink having an increase in osmotic potential, the drawing of solutes into the grape berry is compensated for with an increase in water content of the grape berry (Smart and Coombe, 1983).

In the later part of the grape maturity, an increase took place in phenolic content per freshweight of berries and typically there was an increase in the anthocyanin content per

freshweight of berries. In some cases, mass did decline although this was not the case for all cultivars. Chambourcin in particular had increase in its mass approaching harvest in 2009 (Table 4.2). Norton did not have noticeable decline in berry mass either as it remained rather stable in mass post-veraison in 2009 and showed increase in berry mass until harvest in the following 2010 season (Table 4.2). Since the majority of cultivars saw decline in their berry mass post-veraison during the two seasons included in this study, as shown in Table 4.2, the theory that vascular connections to the grape cluster have ceased has been proposed at times. This however is not the case. While symplatic transport to grape clusters was found to end, apoplastic transport via the xylem continues, a feedback mechanism that includes preventing excessive water accumulation allows for excessive water entry into the berry mesocarp (Keller *et al.*, 2006).The decline in berry mass did not take place with Chambourcin and Norton, suggesting that phloem transport of solutes continued later in grape maturation than with Cabernet Franc and Cabernet Sauvignon.

4.4.2 Organic acids

Tartaric acid is unique to grapes, grapes being part of the *Vitis* genus, in that it accumulates in high levels in the leaves of the plant as well as the fruit (Stafford, 1959). Tartaric acid measured as per berry content was shown to remain stable or increase post-veraison for the majority of the four cultivars in this study in both the 2009 and 2010 seasons (Figure 4.3 and Figure 4.4). In some instances, which can be observed in Figure(s) 4.3 and 4.4, there were some collections post-veraison in which the TA content uncharacteristically declined or had highly variable standard errors than the other collection results for tartaric acid.

The following describes the possible explanation for sudden variability in single collection results in our study. The fact that tartaric is the only organic acid produced in grapes known to be relatively inert, not metabolized by the plant or by microbes during berry ripening means that it should remain in similar concentrations throughout post-veraison except in circumstances where the berry mass has increased significantly. However, like oxalic acid, which is know to be bound in druses within the berry

mesocarp, there is considered to be less free-TA post-veraison as TA becomes predominately in salt form. It is also believed that pH increase in grapes post-veraison is due in part to increased organic acid salts formation (Iland and Coombe, 1988). Although mixing by use of a vortex mixer for two hours was done in our study, there was still the possibility that some organic acids remained bound in salt form with the high level of solutes characteristic to grapes post-veraison.

For organic acids, there was noticeable change after the veraison in the content of individual organic acids in the grapes as well as the titratable acidity measurement of total acids in the grape juice. This is evident on the graphs of all four cultivars in both seasons of this study in which a decline in malate content of the berry was shown by a downward slope of the curve having its maximum content near the onset of veraison (Figure 4.3. and Figure 4.4.). A general decline in the malic acid content from veraison until the date of harvest was typical with the exception being within one month of harvest where a slight increase in malate content per berry was noticed.

Decline in malic acid content per berry might be influenced by climatic factors such as rainfall post-veraison. Water supply as controlled by irrigation treatments has been found to influence malate levels. In Kliewer and Lider (1968), both the shading treatments to grape vines and vines which received irrigation resulted in lower rates of photorespiration of the metabolite malate from the grape berry. Malate declines post-veraison; there is thought to be a shift in the metabolism consisting of mostly primary sugars pre-veraison to organic acids post-veraison (Morrison and Noble, 1990). Heavy shading to vines to increase malic acid levels may actually result in lowering of the MA content. The severe lowering of content through box treatments of clusters resulted in berry MA for the moderately exposed and highly exposed clusters of over 3-fold greater than observed for the box treatments in study conducted in 2000 and 2001 (DeBolt *et al.*, 2008). In a review on the topic of berry growth and development, the author summarizes the decline in malic acid post-veraison as being depending on the factors of respiration, the degradation of enzymes pertaining to malic accumulation, and also being diluted in the berry as berry mass increases (Dokoozlian 2000). With respiration being a factor influencing MA decline, the rapid decline of MA in warm regions is due to MA in grapes

being metabolized during respiration as mentioned in the review on environmental and management practices by Jackson and Lombard (1993).

4.4.3 Climatic influence on grapes

Bloom time has been linked with the degree days leading up to bloom. In our study, time of the end of flowering was nine days in duration from the time of the end of flowering of Chambourcin until the time of the end of flowering for Norton for the 2009 season. In the 2010 season, the end of flowering for all cultivars was documented as being on the same day.

Fruitfulness of cultivars was greater with higher temperatures. The cultivar Thompson Seedless which requires ample light and heat had improved grape maturation (Koblet, 1985). In the controlled temperature study of Kliewer and Torres (1972), cool season cultivars were unable to accumulate their ideal anthocyanin contents if night-time temperatures were above 20° C. Cultivars which accumulated anthocyanins in higher temperatures, 30° C, for example, did not experience loss of ability to accumulate anthocyanins at the higher temperatures (Kliewer and Torres, 1972). The cultivars Chambourcin and Norton displayed the best cultivar performance of the four cultivars in this study. The ability to reach full-coloration is what is used to measure cultivar performance. There was an obvious tier existing between the French hybrid cultivars Chambourcin and Norton and the *vinifera* cultivars of Cabernet Franc and Cabernet Sauvignon; the hybrid cultivars having high anthocyanin content and the *vinifera* having low anthocyanin content. In conclusion, the French hybrids displayed better cultivar performance (Figure(s) 4.1 &4.2). In cultivars which do not tolerate high temperatures as well, a decline in the quality of the fruit can be due to developmental stress that generally increases as temperatures increase. Because of developmental stress and localized climatic conditions, the harvest date for any cultivar varies within a grape growing area (Webb *et al.*, 2007). Both developmental stress and varying harvest dates for cultivars make production of uniform wine quality impossible. Therefore, the cultivars grown in a region should be those which are most ideal to the region, i.e. the vines should have the highest cultivar performance. As in our study, a warm climate in the 2010 grape growing

season resulted in an apparent shrinkage of the harvest window encompassing all four cultivars in our study. This narrowing in which to harvest cultivars grown in a vineyard is not ideal from both the standpoint of having a large harvest to deal with in such a short time as well as the cultivars lacking high cultivar performance being the most likely to suffer developmental stress that leads to inferior wine grape quality.

Vineyard management should be minimal in its use of shading or irrigation regimes to alter the grape phenolic composition. Shading has reduced total phenolic content, flavonol content, and anthocyanin content of grapes intended for use in winemaking (Price *et al.*, 1995). Also, when a study on irrigation was carried out over an entire season declines in flavonol and anthocyanin contents were found for vines which had received irrigation (Kennedy *et al.*, 2002). Also, when considering that a vineyard has received proper management, vines are able to compensate for added stress which may be present at certain times during berry ripening. In confronting stress, grape vines have the capacity to increase their energy expenditure towards their fruit by drawing reserves from old growth further down the vine (Koblet, 1985).

The hardiness of cultivars also needs to be considered for the growing area. When the climate of an area experiences the incident of very low temperatures over a period of several years, such as -20° F, a rare event referred to as a freeze, some cultivars are more susceptible than others and will not survive or receive injury (Becker, 1985). *Vinifera* cultivars are considered to be more susceptible to cold than other species of *Vitis.* In rather extreme hardiness environment in Washington, where minimum temperatures were routinely around - 5° F in the included growing areas, *Vitis vinifera* suffered the most injury, followed by species native to America, while French hybrids were found to have the greatest hardiness of all grape species in the study (Clore *et al.*, 1974).

4.5 Conclusion

4.5.1 Phenolics

This study did find noticeable differences in the accumulation patterns of the cultivars. Such differences included anthocyanins, and total phenolics which were the two groups of phenolics that were quantified in this study.

The anthocyanin accumulation was mostly stable with minimal increases during berry maturity for both *Vitis vinifera* cultivars, Cabernet Franc and Cabernet Sauvignon. Anthocyanin accumulation for the French-American hybrids which included Chambourcin and Norton involved more substantial increases from collection to collection upon the start of grape coloration (Figures 4.1 & 4.2). This divide between *Vitis vinifera* and French-American hybrids was apparent in the graphs for the 2009 (Figure 4.1) and 2010 (Figure 4.2). In such context, these results suggest that the French-American hybrids possess some traits native to America which may allow their cultivar performance to be substantially higher in the Kentucky climate.

Total phenolic accumulation pattern was also different among the four cultivars in this study (Figures 4.1 & 4.2). Both *Vitis vinifera* cultivars mean phenolic contents continued to decline for most collection times post-veraison. Such trend in the phenolic accumulation may explain why the Cabernet Franc and Cabernet Sauvignon cultivars displayed relative stability in their anthocyanin contents as well. The French-American hybrids displayed an increase in their phenolic contents post-veraison as the grapes reached maturity. The increase in phenolics was also matched by a similar pace of increase in the anthocyanin contents of Chambourcin and Norton. The trend of phenolic content increase in the time post-veraison suggests that the increase of the total phenolic content post-verasion could be an indicator of cultivar performance in red wine grape species.

4.5.2 Organic acids

Of the four organic acids quantified, the two primary organic acids of tartaric (TA) and malic (MA) varied noticeably among the cultivars in accumulation pattern. The pattern

recognized in TA was manner of change in TA content per berry post-veraison. For MA, the decline from post-veraison to harvest was examined for any distinction one or more of the cultivars had in comparison to the others.

For tartaric acid, increase, stability, and decline in TA content per berry post-veraison resulted for the four cultivars (Figures 4.3 & 4.4). Chambourcin displayed noticeable increase in post-veraison content per berry for the 2009 and 2010 grape growing seasons. For the *Vitis vinifera* cultivars Cabernet Franc and Cabernet Sauvignon, both were stable post-veraison in their mean TA content in both of the season in this study. Only Norton experienced a noticeable decline in its post-veraison mean TA content. Because TA is not metabolized for use in plant respiration in the manner of MA, either increase or stability is expected for per berry content. Norton was the only cultivar in which noticeable decline of TA content happened in both seasons.

MA content was also different for its post-veraison content per berry depending on the cultivar (Figures 4.3 & 4.4). While decline post-veraison was found for all cultivars included in this study, differences existed in the manner in which the content per berry declined based on the cultivar. In 2009, Cabernet Franc was found to decline but had slight increase at harvest from the collection two weeks prior to harvest. The same sort of increase found at the harvest collection as with Cabernet Franc was found with Chambourcin in 2010. The increase in MA content for Cabernet Franc in 2009 and Chambourcin in 2010 is not explained through decline in berry mass approaching harvest. Decline in berry mass would allow for the MA content per gram fresh weight berries to increase, but would not explain the increase in MA content per berry. All other cultivars declined in both seasons of study and experienced no increase in MA content from the collection before harvest to the harvest collection.

4.5.3 Climatic influence on grapes

Examination of cultivar performance was defined as the ability of the cultivar to achieve full coloration even in high temperatures through anthocyanin accumulation. The cultivars Chambourcin and Norton displayed the best cultivar performance of the four cultivars in this study by maintaining their cultivar performance in both 2009 and 2010.

There was an obvious tier existing between the French-American hybrid cultivars Chambourcin and Norton and the *vinifera* cultivars of Cabernet Franc and Cabernet Sauvignon; the hybrid cultivars having high anthocyanin content and the *vinifera* having low anthocyanin content. In conclusion, the French hybrids displayed better cultivar performance (Figure(s) 4.1 &4.2).

In terms of capability of dealing with stress, it is unclear which cultivar performed best in the Kentucky climate. One can either look at the results of the phenolic group of anthocyanins or make a conclusion of which cultivars had less stress during maturation based on organic acid accumulation pattern. If cultivar performance is also used as an inverse indicator of stress, the French-American hybrids would again have performed better by achieving less stress in both growing seasons than the *Vitis vinifera* cultivars that included Cabernet Franc and Cabernet Sauvingon. There would be no clear discernment of stress when using the accumulation pattern of the primary organic acids of the four cultivars included in this study. When looking at TA, Chambourcin had increase in post-veraison mean TA content for Chambourcin, while both Cabernet Franc and Cabernet Sauvignon remained stable and Norton experienced decline (Figures 4.1 and 4.2). Such a variety of responses for pattern of TA accumulation post-veraison indicates no relationship of TA accumulation to the species of *Vitis*. MA accumulation also displayed no clear relationship when looking at the results of both seasons. All cultivars experienced decline from the high mean MA content around veraison, but the manner in which the decline progressed from collection to collection for the grape maturation post-veraison was different among cultivars and seasons (Figures 4.3 and 4.4).

Although not directly quantified in this study, another measure by which to predict cultivar suitability for the state of Kentucky is through hardiness of grape species. French-American hybrids received far less vine injury than the species of *Vitis vinifera* in the environmental extremes of the northwestern state of Washington in the U.S. (Clore *et al.*, 1974). The importance of success of French-American hybrids in the cold winters of the northwestern U.S. is relative to vineyard success in our state as well. Although not a commonplace event at the present, any time an abnormally cold winter does occur in Kentucky; vines will likely receive detrimental injury due to an untypically cold period of

vernalization for our state. Because of the success of French-American hybrids in colder environments such as the northwestern U.S., it is sensible that hybrids that possess native adaptability traits from their American parentage would do better in untypically cold winters. Such first-hand documentation of vine injury primarily in Cabernet Sauvignon and Cabernet Franc for vines utilized in our study in the experimental plot when in dormancy convinces us that winter injury is less common in the French-American hybrids.

To conclude, cultivar performance seems to be the most certain indicator of cultivar suitability to the state of Kentucky based on the results of this study. In both seasons of this study, Chambourcin and Norton saw substantial increases in anthocyanin content in subsequent collections post-veraison. Neither of the *Vitis vinifera* cultivars, Cabernet Franc or Cabernet Sauvignon, showed any substantial increases in their anthocyanin contents post-veraison. Therefore, the French-American hybrids, Chambourcin and Norton, should be considered more suitable to the Kentucky environment than either of the *Vitis vinifera* cultivars examined in our study.

CHAPTER 5: CABERNET FRANC TREATMENT STUDY

5.1 Introduction

Numerous studies have been done regarding the application of active compounds which improve the properties of grapes at harvest. Such important properties of wine grapes that are worth improving include the coloration of the grape and the phenolic content because both of these are known to be important in wine quality (Cheynier *et al.,* 1998). Individual compounds have been shown to have significant affect on berry coloration and berry phenolic content and on the coloration of the wine made from treated berries. An excellent example is the study of Chervin *et al.* (2004), in which wine made from 5% ethanol treated clusters was found to have darker coloration when compared spectrophotomerically to wine made using the control clusters. In other studies examining ways to improve coloration of grapes, the combination of treatments appeared to act synergistically by causing for a noticeable increase in berry coloration when compared to individual treatments or control treatments (Farag *et al.,* 1992; Delgado *et al.*, 2004).

The active compounds to be investigated in their affectiveness of improving coloration and increasing phenolic content in the cultivar Cabernet Franc in this study by treatment of berry clusters were ABA, benzothiadiazole, ethanol, and ethephon.

Each of these compounds acts in a manner to enhance or accelerate processes of berry maturation that occur around the time of veraison. The berry behaves as a sink as it begins to color and ripen into fruit that could be considered consumable (Coombe, 1987).

ABA has a great affect on gene expression further down the anthocyanin biosynthesis pathway. The gene expression that is increased is UDP glucose-flavanoid 3-o-glucosyl transferase, commonly referred to as UFGT. The study of Jeong *et al.* (2004) showed a spike in UFGT 2-4 weeks post-veraison with ABA applied at the start of veraison.

Benzothiadiazole was shown to enhance anthocyanin biosynthesis in grapevines through a long term affect on the genes responsible for expression of anthocyanin biosynthesis. Benzothiadiazole may affect presence of Chalcone Synthase (CHS), which is

fundamental in the phenylpropanoid conversion to polyketides and resulting compounds, include flavanoids, the group to which anthocyanins and grape phenolics belong. Benzothiadiazole (BTH) applied at the end of veraison has been shown to increase gene expression of CHS (Iriti *et al.,* 2004).

5% aqueous ethanol has been shown to convert to ethylene and further transition to secondary plant metabolites which include the anthocyanin and phenolic products of the phenylpropanoid pathway. There was found to be an increase in the anthocyanin biosyntheses following application at 50 % coloration, also referred to as mid-veraison (El-Kereamy *et al.,* 2002).

Ethephon was found to speed up the anthocyanin biosynthesis process in Crimson Seedless table grapes. However, it was not been shown to increase anthocyanin production in grapes (Jayasena and Cameron, 2009). A higher application than the 300 ppm Ethrel ® used in Jayasena and Cameron (2009) used in an earlier study found evidence of increase in grape anthocyanin content of vines that had received foliar application of ethephon at 1,000 ppm (Weaver and Montgomery, 1974).

When ethanol is used in conjunction with ethephon, it was found that coloration was improved most in the season which was warmest. This suggests that although ethanol may be more affective in cool conditions, its ability to deliver the ethylene precursor into the berry can allow for higher ethylene levels thereby bringing about signaling needed to stimulate anthocyanin biosynthesis in berries (Farag *et al.,* 1992).

Through application of treatment at times which have been used in previous research, the goal will be to verify if application of these active compounds will improve coloration and also increase total phenolic content when compared to control samples in the berries of the Cabernet Franc cultivar. In addition, analysis of organic acids was done to determine whether any of the treatments had influence on important winemaking parameters, namely the grape acidity which can be attributed to organic acid content of the grapes (Banhegyi and Loewus, 2004).

5.2 Materials and Methods

5.2.1 Chemicals and standards

Reagent grade NaOH, Ethanol, HCl, and Tween 80 were purchased from Fisher Scientific, Fair Lawn, NJ. Methanol used in phenolics extraction was purchased from Fisher Scientific, Trinidad. Chlorogenic acid to be used for the phenolic standard was purchased from Sigma-Aldrich, Inc., St. Louis, MO. For organic acid analysis by HPLC, HPLC-grade potassium phosphate monobasic was purchased from Sigma-Aldrich, Inc. Phosphoric acid was purchased from Fisher Scientific for use in organic acid extraction.

Treatments included ABA, benzothiadiazole, ethanol, and ethephon in the first season in 2009. Treatments in the second season in 2010 included benzothiadiazole, ethanol, ethephon, and 5% ethanol with ethephon. Reagent grade ABA (Sigma-Aldrich, Inc.) was applied at two rates: low- 250 ppm ABA, and high- 600 ppm ABA; both rates of ABA also containing the surfactant Tween 70 at 0.1 % (w/v). Benzothiadiazole treatment consisted of using the formulation Actigard (50WG, Syngenta). It was applied at a concentration of 0.3 mM benzothiadiazole, the active ingredient. Ethanol application was using 5% ethanol in DI water. Ethephon treatment at 1000 ppm consisted of using the formulation Florel (Florel Brand Growth Regulator, Rhone-Poulenc Ag Company). 5% ethanol with 1000 ppm ethephon was applied. Control treatments consisted of dipping clusters in DI water.

5.2.2 Plant material

Berry sampling of Cabernet Franc vines that had received treatments was done over two seasons. The grapevines which were only used for treatments and were not sampled at any point in the season except for analysis investigating treatment effects were located in a vineyard at the University of Kentucky Horticulture Research Farm in Lexington, Kentucky, USA. The particular section of the vineyard used for berry sampling was established in 2006 with 2.5 m vine spacing and 2.8 m row spacing. A total of 16 rows composed the plot. In the plot, cultivars were arranged in a generalized random block design. Two vines of a cultivar were adjacent in a row and the cultivar grouping was

repeated in three random places to give six total vines of a cultivar in two rows. In both years of study, vines were pruned by hand to 40 to 50 nodes per vine. No other vine management techniques were practiced besides shoot thinning to 3 to 5 nodes per foot of cordon.

In the first season in 2009, eight vines were used for application of the treatments by dipping three clusters from each vine with one treatment and having all six treatments on every vine. The treatments were ABA 250 ppm (Sigma-Aldrich, Inc.), ABA 600 ppm, 0.3 mM benzothiadiazole (Actigard, 50WG), 5% ethanol, 1000 ppm ethephon (Florel), and H_20 control. Eight replicates were allowed for each treatment but the final number of replicates depended on whether the clusters were sufficiently intact and not consumed by birds. Grape berries were sampled at three times during the season. Grape berries were sampled a month after coloration began, five weeks after start of coloration, and then nine weeks after start of coloration which was considered the harvest date. The precise dates were August 24th for the first collection, September 1st for the second collection, and September 29th for the third collection. In the first collection, neither benzothiadiazole nor ethephon treatment clusters were collected because their application called for a later date. In the second collection, ethephon treatment clusters were not collected because the first application had just been applied a week before and it could not be determined whether treatment with ethephon had allowed full affect of eliciting response in phenolic production in the berry. At each collection date, a cluster of an individual treatment from each vine receiving the dipping application treatments was placed in a separate collection bag to serve as a replicate for that treatment.

In the second season in 2010, six vines were used for application of the treatments by dipping three clusters from each vine with one treatment and having all five treatments on every vine. Treatments were 0.3 mM benzothiadiazole (Actigard, 50WG), 5% ethanol, 1000 ppm ethephon (Florel), 5% ethanol with 1000 ppm ethephon, and H_20 control. ABA 250 ppm (ABA L) and ABA 600 ppm (ABA H) treatments were omitted during the 2010 season due to the ethanol and ethephon treatments having performed on a comparable level in 2009 but costing substantially less which was also a consideration in the practicality of using treatments in a vineyard setting in Kentucky. Grape berries were

sampled at three times during the season. Dipping treatment vines were sampled a month after coloration began, five weeks after coloration began, and then seven weeks after start of coloration which was considered the harvest date. The precise dates were August 20th for the first collection, August 27th for the second collection and September 14th for the third collection. In the first collection neither benzothiadiazole, ethephon, nor 5% ethanol with 1000 ppm ethephon treatment clusters were collected because their application called for a later date. In the second collection, ethephon treatment clusters and 5% ethanol with 1000 ppm ethephon treatment clusters were not collected because the first application had just been applied a week before and it could not be determined whether treatment with ethephon had allowed full affect on eliciting response in phenolic production in the berry. At each collection date, a cluster of an individual treatment from each vine receiving the dipping application treatments was placed in a separate collection bag to serve as a replicate for that treatment.

In addition to treating clusters by dipping the clusters with the individual treatments, in the second season the affect of spraying the entire vine was performed. Five vines selected at random locations in the vineyard were sprayed with 5% ethanol with 1000 ppm ethephon and five vines from random locations in the vineyard were sprayed with DI water control. Three clusters were collected from each vine and the clusters collected from the vine were pooled. Subsampling of the three clusters was used to compose one replicate. The spray application and vine subsampling was done for both 5% ethanol with 1000 ppm ethephon and DI water control treatments. Spray treatment vines were sampled nine weeks after start of coloration which was considered the harvest date and was the same day as the third collection of the dipping treatment clusters.

The clusters of spray treatments were collected at the vineyard on the same date as the third collection of the dipping treatment clusters. For subsampling of three clusters from a vine having received a spray treatment, twenty berries from multiple parts of each cluster were selected and the process was done to create a replicate for the individual spray treatments. This subsampling process was carried out a total of five times for the five vines receiving an individual spray treatment.

The treatment collection was taken back to the lab at the Agricultural Science Center North at the University of Kentucky. The replicates of each treatment were frozen in liquid nitrogen and stored in a freezer at -20°C for berry phenolics analysis and organic acid analysis.

5.2.3 Application regime

Identifying treatments on the vines

For dipping application vines, ribbons to identify treatments were attached to individual clusters. For the ABA applications, which used two different ppm concentrations, different identifying ribbons were used. The other treatments and control clusters also received different identifying ribbons corresponding to each treatment received by the cluster.

In 2010, ribbons were placed at the center of trunk of the spray treatment grape vines. For spray treatment sampling, five vines with 5% ethanol with 1000 ppm ethephon and five vines with DI water control were used. Spraying consisted of using a pump sprayer and dosing to the point of dripping of all foliage, clusters, shoots and cordons of the vine.

Application schedule for treatments

In the first season in 2009 the following application schedule was performed. For ABA, application was done at veraison on the days of July 28th, July 29th , and July 31st by dipping clusters at rates of 250 ppm ABA and 600 ppm ABA on full clusters of Cabernet Franc grapes. For benzothiadiazole treatments, the formulation of benzothiadiazole used was Actigard, and it was applied by dipping clusters at 0.3 mM of the active ingredient Acibenzolar S-methyl, which is 126 ppm concentration of Actigard, over three applications on August 5th, August 12th, and August 24th. 5% aqueous ethanol, which is the same as an amount of 50 mL EtOH per liter of H_2O was applied by dipping berry clusters. Application of the 5% aqueous ethanol was at veraison, it was applied on July 29th, July 31st, and August 3rd. For ethephon, application was done at a much later date than other treatments on August 24th to prevent over-ripening that has been attributed to

ethephon application. Control clusters were dipped in DI water on August 5th at a time considered to be mid-way through application of treatments.

In the second season in 2010 the following application schedule was performed. For benzothiadiazole treatments, the formulation of benzothiadiazole used was Actigard, and it was applied by dipping clusters at 0.3 mM of the active ingredient Acibenzolar S-methyl, which is 126 ppm concentration of Actigard, over three applications on August 2nd, August 9th, and August 21st. 5% aqueous ethanol, which is the same as an amount of 50 mL EtOH per liter of H_2O was applied by dipping berry clusters. Application of the 5% aqueous ethanol was at veraison, it was applied on July 25th, July 27th, and July 30th. For ethephon, application by dipping clusters was done at a much later date than other treatments on August 21st to prevent over-ripening that has been attributed to ethephon application. For 5% ethanol with 1000 ppm ethephon, application followed the same schedule as the ethephon treatments. Clusters were dipped in 5% ethanol with 1000 ppm ethephon on August 21st to prevent over-ripening that has been attributed to ethephon application. The spraying treatment of 5% ethanol with 1000 ppm ethephon was also done on August 21st to prevent over-ripening that has been attributed to ethephon application. Control clusters were dipped in DI water on August 2nd, August 9th, and August 21st at the application dates of the benzothiadiazole treatments which were applied over much of the duration of all of the treatment applications and therefore considered the most representative of all treatments. Spray treatment of the DI water control was done on August 21st to correspond to the application of the 5% ethanol with 1000 ppm ethephon spray treatment.

5.2.4 Berry parameters analysis (pH, titratable acidity, and brix) performed in 2010

Analysis of pH was done by using a pH/mV/°C meter, pH 510 series, Malaysia. The meter was pre-calibrated before use to pH 7.00 with a pH 7.00 buffer solution. For use in the pH measurement of replicates, and when measuring titratable acidity, and brix, the portion of the replicate that was set aside for berry parameters analysis was placed into a sealable plastic bag and the berries were crushed thoroughly.

A small-sized 40 mL beaker was used to hold the juice produced from crushing of the berries in the sealable plastic bag. The pH meter was turned on and thoroughly rinsed before using it to measure the pH of the sample. Measurement involved placing the pH meter directly into the grape juice in the 40 mL beaker. The pH of all three replicates for each treatment was measured in 2010.

After measurement of the pH, titratable acidity was measured. Measurement of titratable acidity followed the method described in Cottrell (1968). The contents of the 40 mL sample beaker were mixed before pouring 10 mL of the juice into a 20 mL graduated cylinder. The volume of 10 mL of sample grape juice was poured into a 125 mL Erlenmeyer flask containing 100 mL of Millipore water. The pH meter was turned on for the duration of the procedure. Using 0.1 N NaOH, the pH was adjusted to an endpoint of 8.2. A 1µL-1,000µL pipette was used to deliver the 0.1 N NaOH into the Erlenmeyer flask. Accuracy was done to the nearest 25 µL of 0.1 N NaOH. The titratable acidity was expressed as grams of titratable acids per liter of juice. Three replicates for every treatment were done for measurement of titratable acidity in 2010.

When complete with titratable acidity measurements, brix of the sample was measured. Measurement of brix used a refractometer (Reichert Scientific Instruments, Buffalo, NY). The refractometer was precalibrated before use. The contents of the sample beaker were thoroughly mixed before applying a drop-sized amount of the grape juice to the prism of the refractometer using a thin plastic bar included with the refractometer. Measurement was done by gazing through the eyepiece into a bright light and estimating the degree brix to the nearest tenth of a degree. Three replicates of the degree brix measurement were done for every treatment in 2010.

5.2.5 Phenolics analysis

Extraction of grape berry phenolics

Phenolics were extracted from the replicates of all of the treatments in each collection. The treatment samples to be analyzed were taken from storage at -20°C. They were allowed to thaw and blotted to remove moisture before analysis.

Samples were prepared in 80% methanol as described in the method for phenolics analysis. Ten grams of berries comprised each sample. The samples were placed in a waring blender with 40 mL of 80% methanol and mixed for eight minutes at low speed. The samples were then filtered using a Whatman #42 filter paper placed in a 1 L Erlenmeyer vacuum flask containing a porcelain funnel connected to a vacuum.

Measurement of grape berry phenolics

Phenolics analysis followed the modified Glories' method procedure in Fukumoto and Mazza (2000). The following steps were done for the phenolics analysis:

1. Dilution of extracted phenolics samples with 5% Methanol to achieve spectrophotomeric readings in the 0.1 to 1 Absorbance range. For treatments samples a dilution factor of 2 was used.

2. Placing 0.25 mL of sample or standard in a small beaker and adding 0.25 mL of 0.1% HCl in 95% ethanol and 4.55 mL of 2% HCl.

3. The solution is mixed and allowed to sit for 15 minutes before reading the absorbance at 280 and 520 nm with a spectrophotometer by placing the solution from the beaker into a 3 mL glass cuvette (Fisher Scientific). Three replicates were done for each of the samples. The absorbance at 280 nm corresponds to the total phenolics content of the sample. The absorbance at 520 nm is used as the anthocyanin content estimate.

4. Standards included chlorogenic acid in 80% MeOH for the total phenolics. The anthocyanin content was obtained by using the anthocyanin extinction constant for Malvin-3-glucoside which has been reported as 28,000 $L \cdot cm^{-1}mol^{-1}$which has been computed using Beer's Law.

5.2.6 Organic acid analysis

Extraction of organic acids

The procedure followed the method described by DeBolt *et al.* (2004). The berries, which had been in the collection bags in a -20°C freezer, were weighed to obtain approximately 5 g fresh weight of berries for each of the replicates of the treatments and the berry number and actual mass to milligram accuracy were recorded. This was ground by mortar and pestle using 5 mL of 0.5 M H_3PO_4 at pH 1.5. The contents of the mortar were transferred to a 2 mL centrifuge tube that was used to hold the slurry. The centrifuge tube was placed onto a rotating mixer for 2 hours to thoroughly protonate the slurry. After the slurry was mixed, the 2 mL microcentrifuge tube was rotated at 14,000 rpm for 3.5 minutes by centrifuge. The spun aliquot was then passed through a syringe containing a 0.45 µm filter (0.45 µm millex-HN syringe driven filter unit, Millipore Corporation) before being delivered by pipette to the glass vials to be placed onto the HPLC autosampler.

Measurement of the organic acids

The organic acid extracts in the glass HPLC vials were placed on forty-well HPLC autosampler trays and run on the HPLC autosampler (Dionex, Ultimate 3000). The column (Prevail organic acid 4.6 x 150 mm, Grace Davidson Discovery Sciences) was maintained at 30°C with an injection temperature of 25°C. Injection volume was 10µL. The mobile phase used in the reversed-phase HPLC analysis was 2.5 mM KH_2PO_4 that had been adjusted to pH 2.5 using H_3PO_4. Flow rate was 1mL of mobile phase per minute under isocratic conditions. Detection in order of elution of oxalic (OA), tartaric (TA), malic (MA), and citric (CA) acids was using a diode array detector with UV absorbance at 210 nm.

5.2.7 Statistical analysis

The R version 2.9.2 statistical computing program was used in the Cabernet Franc treatment study (R 2.9.2, Vienna, Austria). In addition to running code for means, and standard errors for phenolic, anthocyanin, organic acids, and berry parameters data, the

program was also used to create models of climatic data to test for the significance of these factors. The ANOVA summary models of the climatic data used the temperature threshold and year as the two climatic factors investigated. For the entire two year collection of the Cabernet Franc treatment study, phenolics and associated berry parameters data and organic acids and associated berry parameters data were used to create ANOVA summary models of temperature threshold by treatment and year by treatment data to determine the significance of these two climatic factors. For individual collections of the Cabernet Franc treatment study involving the treatments used in both years of the study, temperature threshold and year ANOVA summary models were created to determine significance of these factors on individual collection phenolics and associated berry parameters data and organic acids and associated berry parameters data. In the circumstance where treatments were only used in one year of study, temperature threshold by collection was used to model the affect on phenolics and associated berry parameters data and organic acids and associated berry parameters data for the treatments.

Significant difference in treatment means at each collection were determined by using an unequal N Tukey (Honestly Significant Difference) HSD test. The Tukey HSD test allows for uneven replicates to be used in obtaining the mean values of a given sample, ideally consisting of all replicates, for example, six replicates in 2010. The event of having uneven replicates was the case with replicates that were not able to be utilized for analysis due to unavoidable herbivory by insects and birds, or in cases where maturity level was dramatically different than the other replicates and deemed necessary to exclude.

5.3 Results

5.3.1 Phenolics analysis

Cluster treatments

In the analysis of variance for the entire collection of treatments, including the control, the interaction between treatment and temperature threshold was examined for significance ($p \leq 0.05$). Temperature threshold in this study is defined by the number of days in which a minimum temperature of 20° C was either met or below this number of degrees Celsius; the accumulated temperature threshold days can be observed in Table 5.1. Testing the treatment and year interaction for significance ($p \leq 0.05$) was also performed with the years being 2009 and 2010. For the phenolic content ($p = 0.015$) there was significance due to the treatment. Additionally when the interaction of treatment and year on the phenolic content was investigated, it was found that treatment ($p = 0.013$) and treatment by year ($p = 0.013$) were both significant. Anthocyanin content was highly significant based on treatment ($p = 0.001$). The temperature threshold ($p = .525$) and temperature threshold by treatment interaction ($p = 0.487$) was not significant for anthocyanin content for the entire collection. Also, year had significance on anthocyanin content ($p = 0.002$), thus year was significant for anthocyanin accumulation. Investigation of the significance of treatment and temperature threshold on berry mass of the entire collection revealed that the temperature threshold ($p < 0.001$) was highly significant. Also, when treatment and year were analyzed, the year ($p < 0.001$) was highly significant for berry mass.

For the three individual collections of the Cabernet Franc treatments with the control included, the factors of temperature threshold and year were modeled to examine whether they had influence of phenolic content, anthocyanin content, and grape berry mass ($p \leq 0.05$). In the case of the first collection which was done August 24th in 2009, and August 20th in 2010, the following results of the analysis of variance were obtained. Temperature threshold and year did not have significance on phenolic content with p-values of 0.218. For anthocyanin content, there was no significance of the two factors of

temperature threshold and year with p-values of 0.196. Both factors were highly significant for berry mass ($p < 0.001$).

In collection #2 the same factors of temperature threshold and year were used to find their significance on phenolic content, anthocyanin content, and berry mass. Both the phenolic content ($p = 0.078$) and anthocyanin content ($p = 0.172$) were not significantly influenced by either of the two factors. Both of the two factors were highly significant for the Cabernet Franc berry mass ($p < 0.001$) in collection #2.

In collection #3 the factors of temperature threshold and year were included in analysis of variance models to determine whether they had significance on phenolic content, anthocyanin content, and berry mass. For phenolic content, neither temperature threshold nor year was significant with p-values equal to 0.05006. For anthocyanin content, the temperature threshold and year were determined to be highly significant ($p < 0.001$). Both temperature threshold and year were highly significant with p-values < 0.001 for Cabernet Franc berry mass.

Table 5.1. Temperature thresholds for Cabernet Franc treatments

treatment collection #	temperature threshold[a]	
	2009	2010
1	66	39
2	73	45
3	99	61

[a] Number of days in which the mininum temperature was at or below 20° C. The number is representative of the total applicable days this threshold was attained leading up to the collection. Data was utilized from the University of Kentucky Agricultural Information Center to form number of temperature threshold days.

Table 5.2 Rainfall accumulation in 2009 and 2010

month (December to September) 2008-2009 and 2009-2010	rainfall total[a] (inches of precipitation) 2008-2009	2009-2010
December	6.03	4.03
January	10.35	7.23
February	12.88	8.84
March	15.27	10.06
April	20.06	12.35
May	26.1	22.3
June	31.29	26.89
July	38.86	32.95
August	43.39	33.75
September	49.29	34.36

[a] The number represents inches of rainfall accumulated during the season through the end of the month listed in the row. Data obtained from University of Kentucky Agricultural Weather Center <http://wwwagwx.ca.uky.edu/>.

In the initial collection of 2009, collection #1, taking place approximately 1 month after the start of veraison on August 24th, 2009, treatments had significant affect on the phenolic content based on Tukey's honestly significant difference test (Tukey's HSD test) with $p \leq 0.05$ (Table 5.3). The control and ABA low (250 ppm ABA) were found to be significantly lower in their phenolic content than the ABA high (600 ppm ABA) and 5% ethanol. There were no significant differences in anthocyanin content expressed as mg malvidin-3-glucoside per 100 g FW in the first collection (Table 5.3).

In the first treatment collection of 2010 on August 20th, treatment with ethanol had no significant affect on the phenolic content when compared to the control at the significance of $p \leq 0.05$ using Tukey's HSD test (Table 5.3). The anthocyanin content of ethanol compared to the control was not significant at the level of $p \leq 0.05$ (Table 5.3).

In the second collection, on September 1st, 2009, there were no significant differences in phenolic content due to treatment (Table 5.3). Means of the second collection phenolic content remained similar with the first collection. In evaluating significance of treatment

on anthocyanin content in the second collection, there was no significance due to treatment when using Tukey's HSD test with $p \leq 0.05$ (Table 5.3).

In the second collection on August 27th, 2010, no significant difference in the phenolic content mean of the benzothiadiazole and ethanol treated clusters was found in comparison with the control mean at the significance of $p \leq 0.05$ when using Tukey's HSD test (Table 5.3). The anthocyanin content of ethanol treated clusters was significantly greater in comparison to the control clusters and benzothiadiazole treated clusters.

In the third collection on September 29th, 2009, significance due to treatment was found for both phenolic and anthocyanin content with $p \leq 0.05$ (Table 5.3). The control was significantly lower in its phenolic content than all treatments (Table 5.3). For anthocyanin content, the treatments ABA L and Etp were found to be significantly different than the control, ABA H, BTH, and EtOH (Table 5.3). Denotation of significance using letters for both phenolic content and anthocyanin content can be found in Table 5.3.

In the third collection of 2010 on September 14th, no significant difference was found using Tukey's HSD test at the level of $p \leq 0.05$ for neither the phenolic content nor the anthocyanin content of treated clusters when compared to the control which can be observed in Table 5.3. The observation of the higher anthocyanin mean of the clusters having both EtOH and Etp versus only one of the treatments suggests a possible synergistic effect of the combination of the two active ingredients and this will be included in the sub-chapter of 5.4 Discussion under 5.4.1 Phenolics.

Table 5.3. Treatment influence on Cabernet Franc phenolic content and anthocyanin content

collection date		treatment	phenolic content[a] (mg chlorogenic acid /100 g FW)		anthocyanin content[a] (mg malvidin-3-glucoside/100 g FW)	
2009	2010		2009	2010	2009	2010
8/24/2009	8/20/2010			Collection #1		
		control	230 ± 58 b	269 ± 16 ns	47 ± 12 ns	31 ± 3 ns
		EtOH	417 ± 37 a	255 ± 20 ns	33 ± 9 ns	26 ± 3 ns
		ABA H	452 ± 59 a	NA	41 ± 12 ns	NA
		ABA L	261 ± 30 b	NA	55 ± 8 ns	NA
9/1/2009	8/27/2010			Collection #2		
		control	294 ± 19 ns	260 ± 17 ns	34 ± 9 ns	28 ± 4 b
		BTH	422 ± 104 ns	297 ± 12 ns	35 ± 8 ns	41 ± 4 b
		EtOH	407 ± 64 ns	318 ± 15 ns	27 ± 8 ns	52 ± 5 a
		ABA H	333 ± 52 ns	NA	23 ± 7 ns	NA
		ABA L	280 ± 31 ns	NA	35 ± 5 ns	NA
9/29/2009	9/14/2010			Collection #3		
		control	40 ± 17 c	208 ± 10 ns	34 ± 8 b	78 ± 10 ns
		BTH	160 ± 9 b	187 ± 15 ns	29 ± 11 b	63 ± 15 ns
		EtOH	251 ± 20 b	191 ± 10 ns	45 ± 4 b	93 ± 8 ns
		Etp	230 ± 29 b	258 ± 37 ns	67 ± 5 a	90 ± 8 ns
		EtOH + Etp	NA	255 ± 11 ns	NA	119 ± 18 ns
		ABA H	377 ± 15 a	NA	31 ± 4 b	NA
		ABA L	253 ± 26 b	NA	51 ± 3 a	NA

[a] The mean value ± the standard error. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. In the case of significant difference in mean values, different letters represent that treatments are significantly different from one another. The ranking of treatments is in descending order starting with (a) representing the highest mean values, the term ns used if no treatment content was significantly different at the collection. NA was used where treatments were not applied for the respective season of the study.

Table 5.4. Treatment influence on Cabernet Franc berry mass

collection date		treatment	berry mass[a]	
2009	2010		(g FW)	
			2009	2010
8/24	8/20		Collection #1	
		control	1.197 ± 0.104 ns	1.667 ± 0.000 ns
		EtOH	1.216 ± 0.155 ns	1.738 ± 0.091 ns
		ABA H	0.866 ± 0.096 ns	NA
		ABA L	1.253 ± 0.068 ns	NA
9/1	8/27		Collection #2	
		control	1.076 ± 0.109 b	1.738 ± 0.091 ns
		BTH	1.341 ± 0.096 a	1.722 ± 0.056 ns
		EtOH	1.130 ± 0.081 b	1.683 ± 0.074 ns
		ABA H	0.729 ± 0.087 b	NA
		ABA L	0.971 ± 0.127 b	NA
9/29	9/14		Collection #3	
		control	1.142 ± 0.125 ns	1.648 ± 0.061 ns
		BTH	1.037 ± 0.037 ns	1.528 ± 0.028 ns
		EtOH	1.389 ± 0.139 ns	1.575 ± 0.092 ns
		Etp	1.172 ± 0.141 ns	1.552 ± 0.065 ns
		EtOH + Etp	NA	1.349 ± 0.095 ns
		ABA H	0.916 ± 0.191 ns	NA
		ABA L	1.444 ± 0.222 ns	NA

[a] The mean value ± the standard error. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. In the case of significant difference in mean values, different letters represent that treatments are significantly different from one another. The ranking of treatments is in descending order starting with (a) representing the highest mean values, the term ns used if no treatment content was significantly different at the collection. NA was used where treatments were not applied for the respective season of the study.

In analysis of treatment affect on berry mass expressed as grams per berry, there was no significance in the first and third collections in 2009 using Tukey's HSD test at $p \leq 0.05$ as represented in Table 5.4. Only the second collection benzothiadiazole treatment was significantly different than the other treatments in its berry mass (Table 5.4). In 2010, there was no significant difference found between the treatments and control mean berry mass for all three collections (Table 5.4).

In 2010, brix measurements for treatments to Cabernet Franc clusters were taken to determine any significance that a treatment may have on soluble solids content (Table 5.5). In the first collection there was no significant difference between the control and EtOH treatments using Tukey's HSD test with $p \leq 0.05$. For the second collection, no significant difference was found between the treatments including the control, BTH, and EtOH. For collection #3 taken at the time of harvest, no significance between the control, BTH, EtOH, Etp, and EtOH + Etp treatments was found.

In 2010, pH measurements of Cabernet Franc clusters receiving treatments were recorded to determine if any significance existed between the treatments and control. In collection #1, the EtOH treated clusters were significantly higher in pH than the control when determined by Tukey's HSD test using the level $p \leq 0.05$ (Table 5.5). For the next collection including control, BTH, and EtOH treatments there was no significance in the pH due to treatment. In the collection #3 taken at harvest, no significant difference in the pH measurement due to treatment was found for the control, BTH, EtOH, Etp, and EtOH + Etp treatments.

Table 5.5. Treatment influence on Cabernet Franc brix and pH measurements

collection date 2010	treatment	brix[a] (°Brix)	pH[a] (pH)
8/20/2010		Collection #1	
	control	17.3 ± 0.3 ns	3.28 ± 0.01 b
	EtOH	17.8 ± 0.1 ns	3.32 ± 0.01 a
8/27/2010		Collection #2	
	control	19.3 ± 0.1 ns	3.35 ± 0.03 ns
	BTH	18.9 ± 0.4 ns	3.29 ± 0.02 ns
	EtOH	19.5 ± 0.0 ns	3.36 ± 0.01 ns
9/14/2010		Collection #3	
	control	22.1 ± 0.4 ns	3.57 ± 0.04 ns
	BTH	22.4 ± 0.1 ns	3.53 ± 0.01 ns
	EtOH	21.3 ± 0.9 ns	3.56 ± 0.03 ns
	Etp	22.8 ± 0.2 ns	3.54 ± 0.01 ns
	EtOH + Etp	21.4 ± 0.6 ns	3.60 ± 0.01 ns

[a] The mean value ± the standard error. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. In the case of significant difference in mean values, the letters represent that treatments are significantly different from one another. The ranking of treatments is in descending order starting with (a) representing the highest mean values, the term ns used if no treatment content was significantly different at the collection. Evaluation of treated Cabernet Franc clusters for brix and pH was only done in the second year of study in 2010.

Spray treatments

As a part of the third collection on September 14th, 2010, spray treatments to foliage and grape clusters of Cabernet Franc were done. The two treatments were EtOH + Etp and the control. phenolic and anthocyanin contents of the EtOH + Etp treatment and the control were compared using Tukey's HSD test for significance ($p \leq 0.05$). Both the

phenolic and anthocyanin contents of the two treatments were not significantly different (SEE APPENDICES; Appendix A.5: Cabernet Franc Treatment Study, Table A.5.1).

For the collection of the spray treatments, berry masses of the control and EtOH + Etp were compared using Tukey's HSD test at $p \leq 0.05$. There was no significant difference in berry mass expressed as g per berry between the two treatments. In fact, the grapes of the vines which received the EtOH + Etp spray treatment had a slightly lower mean of 1.783 g per berry than the control with 1.809 g per berry.

In 2010, brix measurements for spray treatments to Cabernet Franc clusters were measured to determine any significance that a treatment may have on soluble solids content. When the control and EtOH + Etp treatments were compared using Tukey's HSD test at the level of $p \leq 0.05$, there was no significant difference in brix measurements.

In 2010, pH was measured for the control and EtOH + Etp spray treatments using Tukey's HSD test for significance using Tukey's HSD test at the level $p \leq 0.05$. As part of the collection taken at harvest, the EtOH + Etp spray treatment had significantly higher pH of 3.52 than the control with pH of 3.63 (SEE APPENDICES; Appendix A.5: Cabernet Franc Treatment Study, Table A.5.2).

Single season treatments analysis of variance

In the case of the cluster dipping treatments ABA H, ABA L, EtOH + Etp, and the spray treatments EtOH + Etp and control, all had only one season of study for their affects on Cabernet Franc grapes. Therefore, analysis of both temperature threshold and collection was possible for all treatments. An anova model was thereby made to determine whether the factor of collection and/or the factor of temperature threshold were significant, at the 0.05 level of significance ($p \leq 0.05$), for Cabernet Franc phenolic content, anthocyanin content, and berry mass. The two factors of collection and temperature threshold are distinct from one another because collection was based solely on the maturity which should have been comparable from season to season while the factor temperature threshold was dependant on the season.

An analysis of variance of the entire season collection which consisted of three total collections of these treatments only applied for one year found the following significant factors at $p \leq 0.05$ for phenolic content, anthocyanin content, and berry mass of the Cabernet Franc grapes. Collection was found to be significant for the phenolic content ($p = 0.005$). For the anthocyanin content, both temperature threshold ($p = 0.016$) and collection by temperature threshold interaction ($p = 0.027$) were found to be significant. For berry mass, the collection, temperature threshold, and collection by temperature threshold interaction were all highly significant with p-values < 0.001.

When comparing the 2009 and 2010 seasons there were different number of temperature threshold at each of the collections which made it possible to determine the significance, $p \leq 0.05$), of the factor temperature threshold on phenolic content, anthocyanin content, and berry mass; the observed difference in both seasons of accumulation of temperature threshold days can be found in Table 5.1. For the third collection, there was more than one temperature threshold for the collection since spray treatments were part of the third collection in 2010 and ABA H and ABA L were treatments for the season of 2009. Therefore, the third collection analysis of variance model included the factor of temperature threshold to determine the significance that it had on phenolic content, anthocyanin content, and berry mass of Cabernet Franc grapes.

In the third collection, when using anova with $p \leq 0.05$, temperature threshold was significant for phenolic content of Cabernet Franc with the p-value < 0.001. Temperature threshold was not significant on the anthocyanin accumulation of the grapes with the p-value of 0.118. For the berry mass of Cabernet Franc, temperature threshold ($p = 0.009$) was significant.

5.3.2 Organic acid analysis

In the analysis of variance for the entire collection of treatments, including the control, the interaction between treatment and temperature threshold was examined for significance with $p \leq 0.05$. Temperature threshold in this study is defined by the number of days in which a minimum temperature of 20° C was either met or below this number of degrees Celsius, for temperature threshold days accumulated at each collection see

Table 5.6. Testing the treatment and year interaction for significance was also performed with the years being 2009 and 2010. For the TA content there was high significance due to temperature threshold ($p < 0.001$) with the level of significance established as $p \leq 0.05$. Additionally when the interaction of treatment and year on the TA content was investigated, it was found that year ($p < 0.001$) was significant but treatment ($p = 0.8381$) did not have significance. For MA content, there was no significance of treatment ($p = 0.191$)and temperature threshold ($p = 0.430$). The treatment and year interaction was significant for the entire collection with both year ($p < 0.001$) and the treatment by year interaction ($p = 0.043$) having had significance. For OA content and CA content, there was no significance due to the factor of treatment ($p = 0.809$, $p = 0.251$, respectfully). Both temperature threshold and year were significant for OA content and for CA content with the respective p-values of 0.026 and < 0.001 for temperature threshold and with p-values < 0.001 for year. Investigation of the significance of treatment and temperature threshold on berry mass of the entire collection revealed that temperature threshold ($p < 0.001$) was significant but that treatment ($p = 0.478$) was non-significant. When treatment and the year were analyzed, the year ($p < 0.001$) was significant for berry mass while treatment ($p = 0.387$) was not significant.

Table 5.6. Temperature thresholds for Cabernet Franc treatments

treatment collection #	temperature threshold[a]	
	2009	2010
1	66	39
2	73	45
3	99	61

[a] Number of days in which the mininum temperature was at or below 20° C. The number is representative of the total applicable days this threshold was attained leading up to the collection. Data was utilized from the University of Kentucky Agricultural Information Center to form number of temperature threshold days.

In collection #1, the factors temperature threshold and year were examined for their significance in Cabernet Franc TA, MA, OA, and CA contents and the berry mass of Cabernet Franc. Both temperature threshold and year were highly significant with p-values < 0.001 on the TA content when included in the analysis of variance model with the level of significance set at $p \leq 0.05$. The same two factors were highly significant for MA content with p-values of 0.004. Both factors were significant ($p \leq 0.05$) on the OA content of Cabernet Franc grapes with p-values < 0.001. The two factors were also significant on Cabernet Franc CA content with p-values of 0.006. Berry mass was highly significant for the two factors with p-values < 0.001.

In collection #2, the factors temperature threshold and year were modeled in anova to determine if they were significant for TA, MA, OA, and CA content and berry mass of Cabernet Franc. The factors of temperature threshold and year were significant for the Cabernet Franc TA content with their p-values of 0.001. Both factors were also significant for the MA content with p-values of 0.016. The two factors were also significant for grape OA content with p-values of 0.009. For CA content, neither temperature threshold nor year were significant with p-values of 0.07. Both the temperature threshold and year ($p < 0.001$, $p < 0.001$) were highly significant on berry mass of Cabernet Franc.

In collection #3, the factors temperature threshold and year were included in the anova model to determine their level of significance on Cabernet Franc TA, MA, OA, and CA content and the berry mass of the grapes. Both temperature threshold and year (p-values < 0.001) were highly significant for TA content of the grapes. Both of the factors were also significant for the MA content ($p = 0.009$). Neither of the factors was significant on the OA content of Cabernet Franc grapes (p-values = 0.614). The two factors were also significant for the CA content (p-values = 0.029). Both temperature threshold and year ($p < 0.001$, $p < 0.001$) were highly significant on berry mass of Cabernet Franc at the level of $p \leq 0.05$.

In the first collection of 2009, for the tartaric acid (TA) content, no significance was found due to treatment using Tukey's HSD test $p \leq 0.05$ as presented in Table 5.7. For

malic acid (MA), no significant difference based on treatment was found ($p \leq 0.05$) as shown in Table 5.7. Oxalic acid (OA) content also showed no significant difference ($p \leq 0.05$), represented in Table 5.8. Citric acid (CA) content was also found to have no significant difference due to treatment ($p \leq 0.05$) as shown in Table 5.8.

In 2010 for the first collection, there was no significant difference between treatments and the control in the TA content per berry when using Tukey's HSD test at $p \leq 0.05$ (Table 5.7). For the MA content of the first collection, there was no significant difference ($p \leq 0.05$) between the control and EtOH clusters due to treatment (Table 5.7). In the first collection, there was no difference in the mean of the OA content between control and EtOH clusters at the significance of $p \leq 0.05$ (Table 5.8). There was also no significant difference in the mean of the CA content of the control and EtOH clusters when using $p \leq 0.05$ (Table 5.8).

In the second collection in 2009, there was no significance due to treatment for the tartaric acid content ($p \leq 0.05$) presented in Table 5.7. Malic acid content had no significance due to treatment ($p \leq 0.05$) as represented in Table 5.7. For oxalic acid content, no significance was attributed to treatment ($p \leq 0.05$) presented in Table 5.8. No significance was found with the citric acid content due to treatment ($p \leq 0.05$) as represented in Table 5.8.

In the second collection for the 2010 season, there was no significance at $p \leq 0.05$ for the TA content mean (Table 5.7). There was no significance with the MA content of the treatments versus the control at $p \leq 0.05$ (Table 5.7). There was also no significance due to treatment for OA content using $p \leq 0.05$ as evident in Table 5.8. There was significant difference ($p \leq 0.05$) of the means for CA content between treatments (Table 5.8).

In the third collection of 2009, EtOH was not analyzed for organic acids in the third collection because of lack of harvestable clusters that received the EtOH treatment. For tartaric acid content, no significant difference due to treatment was found ($p \leq 0.05$) presented in Table 5.7. Table 5.7 also shows lack of significance of treatment on malic acid content per berry ($p \leq 0.05$). Oxalic acid content in the third collection was the only collection and only organic acid where significance due to treatment was noted ($p \leq$

0.05), as presented in Table 5.8. Citric acid content was not significant based on treatment ($p \leq 0.05$) as presented in Table 5.8.

In the third collection during the 2010 season, no significance due to treatment was found for the TA content with $p \leq 0.05$, also evident in Table 5.7. There was no significance due to treatment on MA content at $p \leq 0.05$, also evident in Table 5.7. For OA content, there was no significant difference between treatments and the control with $p \leq 0.05$, also see Table 5.8. There was also no significance due to treatment for CA content at $p \leq 0.05$, also evident in Table 5.8.

Table 5.7. Treatment influence on Cabernet Franc berry tartaric acid content and malic acid content

collection date		treatment	tartaric acid content[a] (mg tartaric acid /berry)		malic acid content[a] (mg malic acid/berry)	
2009	2010		2009	2010	2009	2010
8/24	8/20			Collection #1		
		control	10.49 ± 1.27	5.65 ± 0.21	8.31 ± 1.75	4.06 ± 0.23
		EtOH	9.71 ± 0.89	5.62 ± 0.30	5.79 ± 0.82	3.65 ± 0.33
		ABA H	8.66 ± 0.69	NA	4.93 ± 0.34	NA
		ABA L	8.64 ± 0.42	NA	5.79 ± 0.25	NA
9/1	8/27			Collection #2		
		control	8.63 ± 0.65	5.85 ± 0.43	5.04 ± 0.54	3.02 ± 0.21
		BTH	8.95 ± 1.58	5.52 ± 0.30	6.13 ± 0.19	3.14 ± 0.15
		EtOH	7.87 ± 1.77	5.95 ± 0.21	11.07 ± 4.47	3.29 ± 0.29
		ABA H	6.13 ± 0.18	NA	12.41 ± 0.12	NA
		ABA L	8.90 ± 1.35	NA	3.96 ± 0.54	NA
9/29	9/14			Collection #3		
		control	7.75 ± 0.53	5.59 ± 0.54	1.37 ± 0.23	2.32 ± 0.19
		BTH	8.25 ± 1.07	6.29 ± 0.38	1.13 ± 0.10	2.56 ± 0.24
		EtOH	ND[b]	6.13 ± 0.32	ND	2.02 ± 0.21
		Etp	7.35 ± 0.41	6.85 ± 0.43	2.57 ± 0.19	2.90 ± 0.39
		EtOH + Etp	NA	5.41 ± 0.27	NA	2.57 ± 0.17
		ABA H	8.81 ± 0.65	NA	3.11 ± 0.76	NA
		ABA L	8.81 ± 0.63	NA	3.83 ± 0.54	NA

Table 5.7 (Continued). Treatment influence on Cabernet Franc berry tartaric acid content and malic acid content

[a] The mean value ± the standard error. [b] The terminology ND stands for the ethanol treated clusters that were unable to be harvested. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. No significant differences were found between contents of treatments and the control content for tartaric acid and malic acid. NA was used where treatments were not applied for the respective season of the study.

Table 5.8. Treatment influence on Cabernet Franc berry oxalic acid and citric acid content

collection date 2009	collection date 2010	treatment	oxalic acid content[a] (mg oxalic acid/berry) 2009	oxalic acid content[a] (mg oxalic acid/berry) 2010	citric acid content[a] (mg citric acid/berry) 2009	citric acid content[a] (mg citric acid/berry) 2010
8/24	8/20			Collection #1		
		control	1.47 ± 0.26 ns	0.68 ± 0.05 ns	0.17 ± 0.06 ns	0.26 ± 0.01 ns
		EtOH	1.37 ± 0.25 ns	0.73 ± 0.04 ns	0.06 ± 0.06 ns	0.42 ± 0.09 ns
		ABA H	1.31 ± 0.14 ns	NA	0.15 ± 0.05 ns	NA
		ABA L	1.51 ± 0.20 ns	NA	0.13 ± 0.05 ns	NA
9/1	8/27			Collection #2		
		control	1.44 ± 0.22 ns	0.84 ± 0.03 ns	0.17 ± 0.03 ns	0.35 ± 0.03 a
		BTH	1.50 ± 0.39 ns	0.74 ± 0.05 ns	0.07 ± 0.09 ns	0.16 ± 0.05 b
		EtOH	0.85 ± 0.18 ns	0.85 ± 0.03 ns	0.21 ± 0.06 ns	0.28 ± 0.02 a
		ABA H	0.62 ± 0.10 ns	NA	0.21 ± 0.04 ns	NA
		ABA L	1.62 ± 0.18 ns	NA	0.07 ± 0.07 ns	NA
9/29	9/14			Collection #3		
		control	0.68 ± 0.08 b	0.83 ± 0.08 ns	0.07 ± 0.07 ns	0.17 ± 0.05 ns
		BTH	0.93 ± 0.06 b	0.94 ± 0.06 ns	0.17 ± 0.01 ns	0.22 ± 0.08 ns
		EtOH	ND	0.86 ± 0.06 ns	ND	0.23 ± 0.05 ns
		Etp	1.03 ± 0.12 b	1.03 ± 0.09 ns	0.07 ± 0.04 ns	0.28 ± 0.04 ns
		EtOH + Etp	NA	0.92 ± 0.06 ns	NA	0.19 ± 0.11 ns
		ABA H	1.14 ± 0.17 b	NA	0.28 ± 0.04 ns	NA
		ABA L	1.47 ± 0.20 a	NA	0.29 ± 0.05 ns	NA

Table 5.8 (Continued). Treatment influence on Cabernet Franc berry oxalic acid and citric acid content

[a] The mean value ± the standard error. [b] The terminology ND stands for the ethanol treated clusters that were unable to be harvested. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. In the case of significant difference in mean values, different letters represent that treatments are significantly different from one another. The ranking of treatments is in descending order starting with (a) representing the highest mean values, the term ns used if no treatment content was significantly different at the collection. NA was used where treatments were not applied for the respective season of the study.

Table 5.9. Treatment influence on Cabernet Franc organic acid sample berry mass

collection date		treatment	berry mass[a] (g FW)	
2009	2010		2009	2010
8/24	8/20		Collection #1	
		control	1.253 ± 0.118 ns	1.991 ± 0.065 ns
		EtOH	1.323 ± 0.110 ns	1.974 ± 0.078 ns
		ABA H	0.964 ± 0.090 ns	NA
		ABA L	1.264 ± 0.070 ns	NA
9/1	8/27		Collection #2	
		control	1.108 ± 0.095 ns	1.751 ± 0.037 ns
		BTH	1.266 ± 0.177 ns	1.605 ± 0.068 ns
		EtOH	1.011 ± 0.146 ns	1.797 ± 0.050 ns
		ABA H	0.987 ± 0.100 ns	NA
		ABA L	1.095 ± 0.227 ns	NA
9/29	9/14		Collection #3	
		control	0.977 ± 0.047 b	1.811 ± 0.096 ns
		BTH	0.917 ± 0.125 b	1.691 ± 0.054 ns
		EtOH	ND[b]	1.707 ± 0.063 ns
		Etp	0.910 ± 0.042 b	1.886 ± 0.058 ns
		EtOH + Etp	NA	1.509 ± 0.108 ns
		ABA H	0.712 ± 0.079 b	NA
		ABA L	1.491 ± 0.138 a	NA

Table 5.9 (Continued). Treatment influence on Cabernet Franc organic acid sample berry mass

[a] The mean value ± the standard error. [b] The terminology ND stands for the ethanol treated clusters that were unable to be harvested. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. The ranking of treatments is in descending order starting with (a) representing the highest mean values, the term ns used if no treatment content was significantly different at the collection. NA was used where treatments were not applied for the respective season of the study.

Berry mass recorded for the organic acid analysis was analyzed for significance to treatment using Tukey's HSD test with $p \leq 0.05$ for the 2009 season (Table 5.9). In the third collection, ABA L had a value significantly different than the other treatments and the control with a mean of 1.491 g per berry (Table 5.9). In 2010, there was no significant difference ($p \leq 0.05$) in berry mass of treatments in comparison to the control (Table 5.9).

Spray treatments

As a part of the third collection on September 14th, 2010, spray treatments to foliage and grape clusters of Cabernet Franc were done. The two treatments were EtOH + Etp and the control. TA, MA, OA, and CA contents of the EtOH + Etp treatment and the control were compared using Tukey's HSD test for significance ($p \leq 0.05$). The contents for TA, MA, OA, or CA of the two treatments were not significantly different (SEE APPENDICES; Appendix A.5: Cabernet Franc Treatment Study, Table A.5.3 and Table A.5.4). For the organic acid content, only mean MA content was greater for EtOH + Etp than the control mean (SEE APPENDICES; Appendix A.5: Cabernet Franc Treatment Study, Table A.5.3).

For the collection of the spray treatments, berry masses of the control and EtOH + Etp were compared using Tukey's HSD test at $p \leq 0.05$. There was no significant difference in berry mass expressed as g per berry between the two treatments. In fact, the grapes of the vines which received the EtOH + Etp spray treatment had a slightly lower mean at 1.662 g per berry FW than the control having a mean of 1.691 g per berry FW (SEE APPENDICES; Appendix A.5: Cabernet Franc Treatment Study, Table A.5.4).

In 2010, titratable acidity for spray treatments to Cabernet Franc clusters were measured to determine any significance that a treatment may have on titratable acidity expressed by total grams of acids/L grape juice. When the control and EtOH + Etp treatments were compared using Tukey's HSD test at the level of $p \leq 0.05$, there was no significant difference in titratable acidity measurements (SEE APPENDICES; Appendix A.5: Cabernet Franc Treatment Study, Table A.5.3).

In 2010, pH was measured for the control and EtOH + Etp spray treatments using Tukey's HSD test for significance using Tukey's HSD test at the level $p \leq 0.05$. As part of the collection taken at harvest, the EtOH + Etp spray treatment had significantly higher pH of 3.63 than the control having the pH 3.52 (SEE APPENDICES; Appendix A.5: Cabernet Franc Treatment Study, Table A.5.2).

Single season treatments analysis of variance

In the case of the cluster dipping treatments ABA H, ABA L, EtOH + Etp, and the spray treatments EtOH + Etp and control, all had only one season of study for their affects on Cabernet Franc grapes. Therefore, analysis of both temperature threshold and collection was possible for all treatments, see Table 5.6 for the accumulation of temperature threshold days. An anova model was thereby made to determine whether the factor of collection and/or the factor of temperature threshold were significant for Cabernet Franc TA, MA, OA, and CA content, and berry mass ($p \leq 0.05$). The two factors of collection and temperature threshold are distinct from one another because collection was based solely on the maturity which should have been comparable from season to season while the factor temperature threshold was dependant on the season.

An analysis of variance of the entire season collection which consisted of three total collections of these treatments only applied for one year found the following significant factors ($p \leq 0.05$) for TA, MA, OA, and CA content, and berry mass of the Cabernet Franc grapes. Temperature threshold ($p < 0.001$) was highly significant and collection ($p = 0.007$) was found to be significant for the TA content. For the MA content, collection was found to be highly significant with the p-value of 0.001. Both temperature threshold ($p = 0.007$) and collection ($p = 0.026$) were significant on the OA content of Cabernet Franc. For CA content, collection was found to be significant with the p-value of 0.028. For berry mass, the collection and temperature threshold were highly significant with p-values < 0001.

When comparing the 2009 and 2010 seasons there were different number of temperature threshold at each of the collections which made it possible to determine the significance of the factor temperature threshold on TA, MA, OA, and CA content, and berry mass. For the third collection, there was more than one temperature threshold for the collection since spray treatments were part of the third collection in 2010 and ABA H and ABA L were treatments for the season of 2009. Therefore, the third collection analysis of variance model included the factor of temperature threshold to determine the significance ($p \leq 0.05$) that it had on TA, MA, OA, and CA content, and berry mass of Cabernet Franc grapes.

In the third collection, when using anova with $p \leq 0.05$, temperature threshold ($p = 0.01$) was significant for TA content of Cabernet Franc. Temperature threshold ($p = 0.409$) was not significant on the MA accumulation of the grapes. Temperature threshold was not significant on OA content with its p-value of 0.054. For CA content, temperature threshold was not significant for Cabernet Franc grapes with its p-value of 0.756. For the berry mass of Cabernet Franc, temperature threshold ($p = 0.003$) was significant.

5.4 Discussion

5.4.1 Phenolics

For all treatments a significant increase in the phenolic content when compared to the control was found in the harvest collection of 2009. There have been previous studies that explain why this is the case.

For benzothiadiazole (BTH), the phenylalanine amino acid content of grape leaves treated with BTH was higher than the control grapes. The strengthening of cell walls through the contribution of lignin by increased activity of the phenylpropanoid pathway is one of the physiological activities which takes place with BTH treatment due to BTH serving as functional analog of plant salicylic acid (Iriti *et al.,* 2005). An increase in the phenylpropanoid pathway activity of the plant would not be limited to lignin formation but would also increase other phenolics classes. This would explain some of the increase over the control's phenolic content in the grapes that had received BTH treatment in our study (Table 5.3).

For treatment with ABA and sucrose, there was increase in the total phenolic content of berry disks when investigated. There was also an increase in total phenolics with ABA only (Pirie and Mullins, 1976). In our study, there was significant increase in the phenolic content of berries treated with ABA when compared to the control using Tukey's HSD test with $p \leq 0.05$ (Table 5.3). Our method of delivery via dipping of clusters with ABA treatment was different than treatment of berry slices with a concentration of ABA which was approximately 5.3 ppm. This ABA concentration was much less than the concentration utilized in our study but it was delivered directly onto an excised berry slice which would mean that diffusion would have been much more efficient than an application applied to the exterior of the berry clusters. For the low ABA concentration at 250 ppm, there was significant increase in anthocyanin content at the 2009 harvest when compared to the control at $p \leq 0.05$ (Table 5.3).

Ethephon application during a previous study resulted in accelerated ripening as measured through the soluble solids content of the grape; furthermore, increases in must

color of at least 10% were found for both 250 ppm and 500 ppm applications of ethephon. There was an increase in mean phenolic content at both concentrations of ethephon, however neither concentration resulted in significant difference in phenolic content in comparison to the control at the 5% level of significance. Application in the study of Powers *et al.* (1980) was by spray treatment which may not have been as thorough in contacting the berry exocarp as our method of application which involved dipping of the berry clusters. In our study, there was significant increase in phenolic content of the ethephon treated grapes when compared to the control in 2009 (Table 5.3). Also, there was significant increase when compared to the control ($p \leq 0.05$) in the anthocyanin content of clusters treated with ethephon at harvest in 2009 (Table 5.3).

Ethanol (EtOH) results have found consistence from season to season on improvement of coloration in grapes when compared to the control. The grape cultivar used in the study of Chervin *et al.* (2004) was Cabernet Sauvignon grafted onto Richter 110 and vines of the study were grown in a non-irrigated vineyard. A possible correlation with increased titratable acidity in EtOH treated vines was suggested to increase absorbance readings at the 520 nm wavelength used to measure coloration of wines made from three vintages. For all three vintages when measuring coloration of wine, a noticeable increase in the mean O.D. for EtOH treated vines was found when comparing to the control mean O.D.. In our study, during the 2009 season EtOH treatment phenolic content increased significantly when compared to the control mean at $p \leq 0.05$ (Table 5.3). In our study, there was no such increase in mean titratable acidity of EtOH treatments when compared with the control mean titratable acidity (Table 5.10). In fact, titratable acidity measurements in 2010 resulted in mean titratable acidity of EtOH treatments 6.30 g acidity/L grape juice and the control mean of 6.51 g acidity/L grape juice at the harvest collection for Cabernet Franc treatments.

Table 5.10. Treatment influence on Cabernet Franc titratable acidity

collection date 2010	treatment	titratable acidity[a] (g titratable acids/ L juice)
8/20/2010		Collection #1
	control	8.13 ± 0.56 ns
	EtOH	7.30 ± 0.46 ns
8/27/2010		Collection #2
	control	7.98 ± 0.87 ns
	BTH	8.33 ± 0.08 ns
	EtOH	6.65 ± 0.25 ns
9/14/2010		Collection #3
	control	6.51 ± 0.63 ns
	BTH	6.20 ± 0.50 ns
	EtOH	6.30 ± 0.24 ns
	Etp	7.09 ± 0.81 ns
	EtOH + Etp	5.76 ± 0.41 ns

[a] The mean value ± the standard error. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. The term ns was used because no treatment content was significantly different at the collection. Evaluation of treated Cabernet Franc clusters for titratable acidity was only done in the second year of study in 2010.

An ethanol + ethephon (EtOH + Etp) treatment was applied to vines in 2010 to investigate whether the delivery of ethephon, an ethylene precursor, by ethanol improved the ability for ethephon to change the grape phenolics accumulation. The climate of 2010 was quite ideal to test this noticed synergy of the combination of EtOH and Etp in anthocyanin biosynthesis of the berries. Such a synergy related to berry anthocyanin content was noticed in the study of Farag *et al.* (1992) where the combination did better than either EtOH or Etp applied individually to cranberries. In our study, although not significant in increase in phenolic content over the control ($p \leq 0.05$), the EtOH + Etp treatment mean phenolic content was greater than the mean phenolic content of the control (Table 5.3). The same was true for anthocyanin content, although not significant at $p \leq 0.05$, the mean anthocyanin content of 119 mg m-3-g/100 g FW was greater than

the control mean of 78 mg m-3-g/100 g FW (Table 5.3). Another plausible treatment that might be considered is using ethylene precursors to elevate ethylene hormone levels in combination with ABA, but ABA might remain at lower levels with the elevation of ethylene in the grape vine (Coombe and Hale, 1973).

Interestingly, the berry size was not found to be higher in 2009 which saw an increased rainfall during the growing season compared to the 2010 growing season (Table 5.4). Some regions, such as the Mediterranean, and in the U.S., Napa Valley in California, rely heavily on rainfall primarily accumulated during the winter months which is capable of storage in the vineyard soils. A potential explanation to this rather complex association of climatic factors, such as rainfall, with the berry size during grape development is that berry size could be profoundly influenced by water-storage capability of soil and therefore early-season rainfall (Smart and Coombe, 1983). In 2010, rainfall accumulation in the early season was very similar to the early-rainfall in 2009 (Table 5.2). Early grape development is characterized by a period of rapid cell devision prior to cell-expansion later in the season. Often, fruit size has been known to be determined by initial cell-division rather than cell-elongation, as noted in a review by Dokoozlian (2000), which could account for the greater berry mass in 2010 than in the 2009 season, although the 2009 season did have more total accumulated rainfall, see Table 5.2 for rainfall accumulation in 2009 and in 2010. In grapes, this result was found by Smart and Bingham (1974), where the observation that water-stressed grapes earlier in the grape season ,which also possessed slower rate of cell-division than properly watered grapes, produced grapes with low mass at maturity compared with the properly watered grapes serving as the control. Regarding precipitation, excessive precipitation can delay the ripening process in grapes, particularly in Stages I and II (Jackson and Lombard, 1993). A concise review on berry development highlight the major stages of development, with the first two stages characterized by rapid cell division in stage 1 and lag phase in stage 2 (Dokoozlian, 2000).

A decline in berry mass between the second and third collections in our study was found for treatments contained in both of these collections: control, EtOH, BTH (Table 5.4). The theory that vascular connections to the grape cluster have ceased has been proposed

at times. This however is not the case. While symplastic transport to grape clusters was found to end, apoplastic transport via the xylem continues, a physiological feedback mechanism which includes preventing excessive water accumulation that allows for water to never even enter the berry mesocarp (Keller *et al.*, 2006).

Research of treated grapes has revealed changes in berry mass in comparison with the control. An earlier study found that ethephon at higher levels of 500 ppm and 1,000 ppm increased berry mass of vines receiving the ethephon treatment when compared to berries of the control vines. When berry mass has been found to increase, this is known to affect ripening indicators such as the sugar content of the berries, but no significant change in sugar content was found. Furthermore, the high level ethephon sprays on vines post-veraison that increased the berry mass were found to create more intense coloration to clusters than found with the control vines (Weaver and Pool, 1971). Another study showed higher berry mass for all ethephon treatments done at weekly intervals except for the first two applications done prior to veraison. As well as increasing the berry mass in this study, the benefit found with all ethephon treatments was the decline in mass of the second crop clusters on the treated vines. The overall conclusion by the authors was that timing of ethephon application will differ based on the intend uses, such as increasing the average berry mass or reducing second crop yield, but this study seems to indicate an overall benefit from ethephon application (Szyjewicz and Kliewer, 1983).

In our study, GA_3 was not considered for use in improving the quality of Cabernet Franc grapes. The potential idea of using gibberellic acid (GA_3) to improve coloration of grapes is one that seems to not be supported by research in grapes, and in fact, use of GA_3 in wine grapes has some disadvantages. One disadvantage for its use in thinning, in reality the opening, of clusters to prevent rotting is that rudimentary ovaries stayed attached to the pedicel which would create less uniformity to the clusters. Another terminology for assorted rudimentary clusters and normally developed clusters is hen and chicks commonly used to describe grape clusters containing large and much smaller grapes. Average normal berry weight was higher for the Thompson Seedless and Zinfandel vines receiving GA_3 treatment, but cluster weight was lower than the control cluster weight due to the presence of rudimentary ovaries on the GA_3 treated clusters

(Miele *et al.*, 1978). Another study found a lack of support in the uses of GA_3 to improve grape maturity and potentially the grapes value for use in winemaking. In the study, a thinning response was not observed when applying GA_3 at bloom which seems to be the logical time to apply a treatment to affectively thin the clusters. Also, the study did not find any increase in fruit maturity which would not make GA_3 a good candidate for improving the quality of grapes at harvest in regions having unfavorable conditions for grapes to reach desired maturity (Kasimatis *et al.*, 1979). In the study conducted by Weaver (1975), the observation of increased pedicel length, which is to say the individual connection of each berry to the entire peduncle, could create the effect of cluster loosing even though no berry thinning was actually done. GA_3 may be useful in the aspect of maintaining grape integrity for better handling post-harvest, but use of GA_3 for the purpose of enhancing anthocyanin accumulation is not one of its traditional uses (Jackson and Lombard, 1993).

5.4.2 Organic acids

Tartaric acid is unique to grapes, grapes being part of the *Vitis* genus, in that it accumulates in high levels in the leaves of the plant as well as the fruit (Stafford, 1959). Tartaric acid measured as per berry content was shown to remain stable or increase post-veraison in both seasons where, for each treatment, the mean TA content per berry only varied 2-3 mg in 2009 and by less than 1mg in 2010 (Table 5.9). The fact that tartrate is the only organic acid produced in grapes known to be relatively inert during berry ripening means that it remains in similar concentrations throughout post-veraison except in circumstances where the berry mass has increased significantly.

For organic acids, there was noticeable change after the veraison in the content of individual organic acids in the grapes as well as the titratable acidity measurement of total acids in the grape juice. A good example is a notable decline in the total acidity from the first collection to the two collections following the first for all applicable treatments (Table 5.10). One organic acid which saw noticeable decline in its content was MA. In all applicable treatments, a decline in the malic acid took place in one collection from the next. Generally, the common-held belief is that malic acid is

metabolized later in the season. Malic acid declines post-veraison when it is used as a respiratory substrate. There is shift in the respiratory substrate from sugar metabolism to acid metabolism (Morrison and Noble, 1990). In a review on the topic of berry growth and development, the author summarizes the decline in malic acid post-veraison as being depending on the factors of respiration, the degradation of enzymes pertaining to malic accumulation, and also being diluted in the berry as berry mass increases (Dokoozlian, 2000). With respiration being a factor influencing MA decline, the rapid decline of MA in warm regions is due to MA in grapes being metabolized during respiration as mentioned in the review on environmental and management practices by Jackson and Lombard (1993).

Stomatal measurements of grape leaves have shown that stomata of plants having grape clusters developing on them have lower diffuse resistance, meaning they are more open (Smart and Coombe, 1983). This would then seem to indicate the increased photosynthesis which is needed for grape vines growing fruit. In fact, grapes vines grown for fruit production are known to possess one of the highest maximum photosynthesis rates of C_3 plants being not far below maximum photosynthetic rates of C_4 plants (Smart and Coombe, 1983). However, this would also imply that greater rates of respiration would exist because of the increased energy requirements. Interestingly, ABA has increased the diffuse resistance of grape leaves through greater closure of the stomata (Smart and Coombe, 1983). The possibility that ABA causes for an increase in available plants sugars and metabolite by-products to grape clusters is potentially the mechanism whereby the hormone alters organic acids in comparison with the control such as noticed in a higher mean for malic acid content for ABA treatments compared to the control mean for the third collection taken at harvest (Table 5.7). Furthermore, the compound ethephon, which serves as an ethylene precursor could indeed have similar physiological affects on the grape vine due to its disputed role as contributing to accelerated ripening of grapes as mentioned in Jayasena and Cameron (2009).

5.4.3 Climatic influence on grapes

Fruitfulness of cultivars was greater with higher temperatures. The cultivar Thompson Seedless which requires ample light and heat had improved grape maturation (Koblet, 1985). Because Thompson Seedless has more fruitfulness in high temperature and light; this cultivar prefers warmer climates. The ability to reach full-coloration is what is used to measure cultivar performance. Cabernet Franc did not attain high coloration without the use of treatments in both years of this study. Cabernet Franc did not achieve good coloration which argues against the statement that it has good cultivar performance and is supportive of its classification as a cool season cultivar. Treatments were found to improve the anthocyanin content of Cabernet Franc in both seasons with substantial improvements in the mean anthocyanin content for most treatments in the 2010 season (Table 5.3).

Conventional techniques at improving phenolic contents of grapes do not work ideally. Therefore, in the effort to improve coloration and maintain phenolic levels equal or greater than the Cabernet Franc control, treatments were used in this study. Vineyard management should be minimal in its use of shading or irrigation regimes to alter the grape phenolic composition. Shading has reduced total phenolic content, flavonol content, and anthocyanin content of grapes intended for use in winemaking (Price *et al.*, 1995). Also, when a study on irrigation was carried out over an entire season declines in flavonol and anthocyanin contents were found for vines which had received irrigation (Kennedy *et al.*, 2002). Also, when considering that a vineyard has received proper management, vines are able to compensate for added stress which may be present at certain times during berry ripening. In confronting stress, grape vines have the capacity to increase their energy expenditure towards their fruit by drawing reserves from old growth further down the vine (Koblet, 1985).

5.5 Conclusion

5.5.1 Phenolics

Whether treatments were a success is being based on whether mean anthocyanin and total phenolic contents were higher than the mean contents of the control at the third collection corresponding to the time of harvest, but not necessarily found to be significantly higher than the control. Greater mean contents of anthocyanins and phenolics were the case for the ethephon and ABA L treatments both found to be significantly different than the control treatment means in 2009. EtOH treatment means of anthocyanin and phenolic contents were also higher, although not significantly different, than the control means in 2009. ABA 250 ppm (ABA L) and ABA 600 ppm (ABA H) treatments were omitted during the 2010 season due to the ethanol and ethephon treatments having performed on a comparable level in 2009 but costing substantially less which was also a consideration in the practicality of using treatments in a vineyard setting in Kentucky. The ethephon (Etp) and the EtOH +Etp treatments had higher mean contents of anthocyanins and phenolics although their means were not significantly different than the control means in 2010 (Table 5.3). Of the BTH, EtOH, and Etp treatments applied in both seasons, the treatment Etp was affective in raising the anthocyanin and phenolic mean contents beyond the means for the control at harvest in both the 2009 and 2010 seasons although not significantly different from the control means in 2010, also evident in Table 5.3.

The success of the treatments in elevating anthocyanin content in vines in this study supports the use of treatments to improve coloration in Cabernet Franc. Because of its performance in the 2009 and 2010 seasons, ethephon is the best candidate for establishing good coloration over multiple growing seasons in the state of Kentucky. Whether ethephon performs more consistently in face of the climatic changes that take place from season to season in our state than the other treatments included in this study has yet to be determined. Other reasonable candidates for improving coloration which were inexpensive and resulted in increased anthocyanin and phenolics means compared to the control means are EtOH in the 2009 season and the EtOH + Etp combination treatment in the 2010 season.

5.5.2 Organic acids

Organic acids were examined for each of the treatments in this study primarily to see what affect, if any, treatment had on organic acid content of the Cabernet Franc berries. The findings suggest no significant affect of treatment on the primary organic acids found in grapes (Table 5.7). The primary organic acids of Cabernet Franc were Tartaric acid (TA) and Malic acid (MA). The two other less abundant organic acids quantified in this study were oxalic acid (OA) and citric acid (CA). Only one incidence of significant difference for each of these organic acids was found in both seasons. The one incidence for each is apparent in Table 5.8 for the incidence of significance due to treatment for OA at harvest in 2009, and for the incidence of significance due to treatment for CA at the second collection in 2010. In conclusion, the lack of significant differences of the treatments and control in the organic acid contents per berry implies that no form of correlation between treatments aimed at improving berry coloration in Cabernet Franc and the per berry contents of TA, MA, OA, CA. In particular, TA and MA, the primary organic acids of Cabernet Franc grapes did not change significantly in their per berry content due to the treatment applied.

5.5.3 Climatic influence on grapes

The observation that the Cabernet Franc control did not affectively accumulate anthocyanins in comparison to the treatment anthocyanin contents in either the 2009 or the 2010 season supports the opinion that Cabernet Franc does not possess high cultivar performance as defined by a cultivars ability to accumulate anthocyanins in berries in high temperatures. Commonly, Cabernet Franc is thought of as a cool-season cultivar.

In order to address viticultural problems in attaining full coloration of Cabernet Franc in the environment of Kentucky, a method by which to do this is needed in order to address the issue in already established vineyard sites growing the Cabernet Franc cultivar. Both irrigation and shading have been found to have adverse affects on phenolic and anthocyanin contents of grapes, see Price *et al.* (1995) and Kennedy *et al.* (2002), respectively in regard to the topic of the study. Therefore, the method of treating vines was used in this study and found to be affective in raising the mean anthocyanin contents

for treatment clusters in comparison to control clusters. Treatments, such as ethephon, the treatment found most affective in the improvement of both total phenolic content and anthocyanin content in our study, should be considered for the purpose of improving coloration in Cabernet Franc in the Kentucky environment.

CHAPTER 6: CONCLUSION

After examination of the phenolic composition of the five wine varietals, it was apparent that each is unique in its phenolic profile of tannin, anthocyanin, and Fe-reactive phenolics. The research also supports the linkage of sensorial ratings to wine phenolic quantification. Astringency rating, color rating, and overall acceptability were all strongly correlated to their associated phenolic groups. It is for this reason that the study of cultivar phenolic and anthocyanin accumulation was done the following 2009 and 2010 growing seasons to address suitability of red wine grape cultivars to this region. Furthermore, a study was carried out during the two grape growing seasons of treatment ability to improve coloration and phenolic content of Cabernet Franc, the varietal that had the lowest anthocyanin content in the wine phenolics research.

The trend of phenolic content increase post-veraison in the cultivar study appears to relate to the ability of cultivars to achieve full-coloration, also termed cultivar performance, in the Kentucky environment. In 2009 and 2010 the French-American hybrids of Chambourcin and Norton were able to accumulate high levels of anthocyanins compared to the *Vitis vinifera* L. cultivars Cabernet Franc and Cabernet Sauvignon. Phenolic contents and anthocyanin contents were similar per cultivar in both seasons with only a maximum change of one rank in mean contents between cultivars. Additionally, trendlines of each cultivars were very similar from one season to the next season; the trendlines of the French-American hybrids were comparable and the trendlines of *Vitis vinifera* cultivars were alike. Due to the decline in phenolic content of the *Vitis vinifera* cultivars post-veraison, the stability of their anthocyanin trendlines seems logical. On the other hand, the French-American hybrids displayed increases in phenolic content post-veraison with concurrent increase in their anthocyanin contents. The trend of phenolic content in the time post-veraison supports the level of cultivar performance for the red wine grape cultivars included in the study.

As displayed in the cultivar study, it became apparent that Cabernet Franc did indeed need help attaining full-coloration in the climatic conditions of this region. Previously documented viticultural practices aimed at improving the phenolic composition of grapes,

such as irrigation and shading have adverse affects. The success of treatments in the study of improving coloration and phenolic content supports the use of treatments to improve coloration of Cabernet Franc in Kentucky. Cabernet Franc did not attain as high of anthocyanin-attributed coloration without the use of treatments in both seasons of this study. Significantly greater mean contents of anthocyanins and phenolics were found for 250 ppm ABA (ABA L) and ethephon in 2009. However, ABA treatments were eliminated from further study in 2010 due to extreme cost at that time in using the plant hormone to treat acreages of wine grapes in the commercial setting. Of the benzothiadiazole, ethanol, and ethephon treatments used in both seasons, ethephon was affective in raising anthocyanin and phenolic means beyond the control means in both seasons of study, although ethephon and control means were not significantly different in the 2010 growing season. Whether ethephon performs more consistently than the other treatments in the study has yet to be determined. Other candidates for improving coloration that were also inexpensive are ethanol, achieving a higher anthocyanin and phenolic mean contents than the control in 2009, and the combination treatment of ethanol with ethephon which also elevated the anthocyanin and phenolic mean contents above the control in 2010.

APPENDICES

Appendix A.3: Wine Phenolics

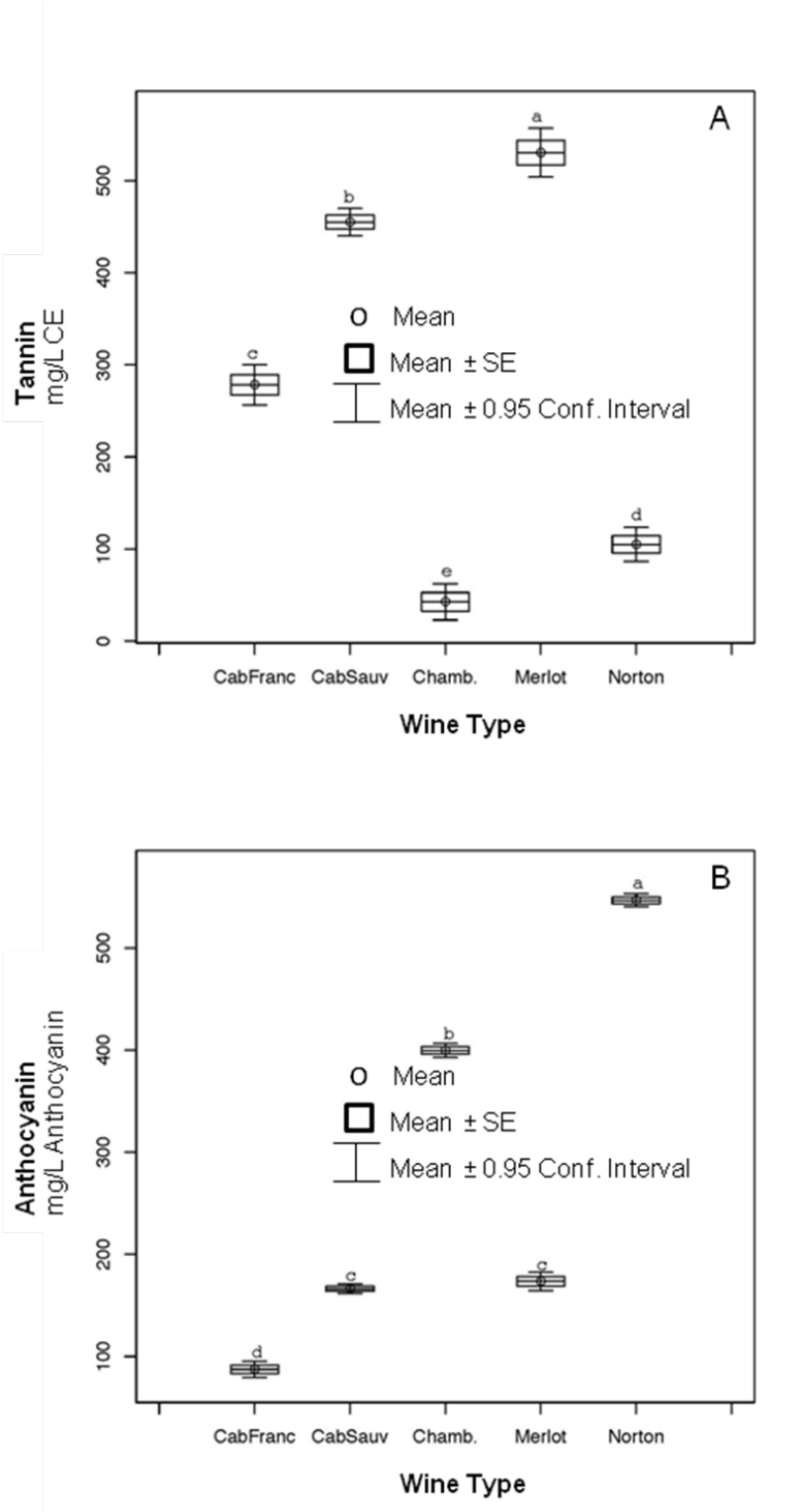


Figure A.3.1

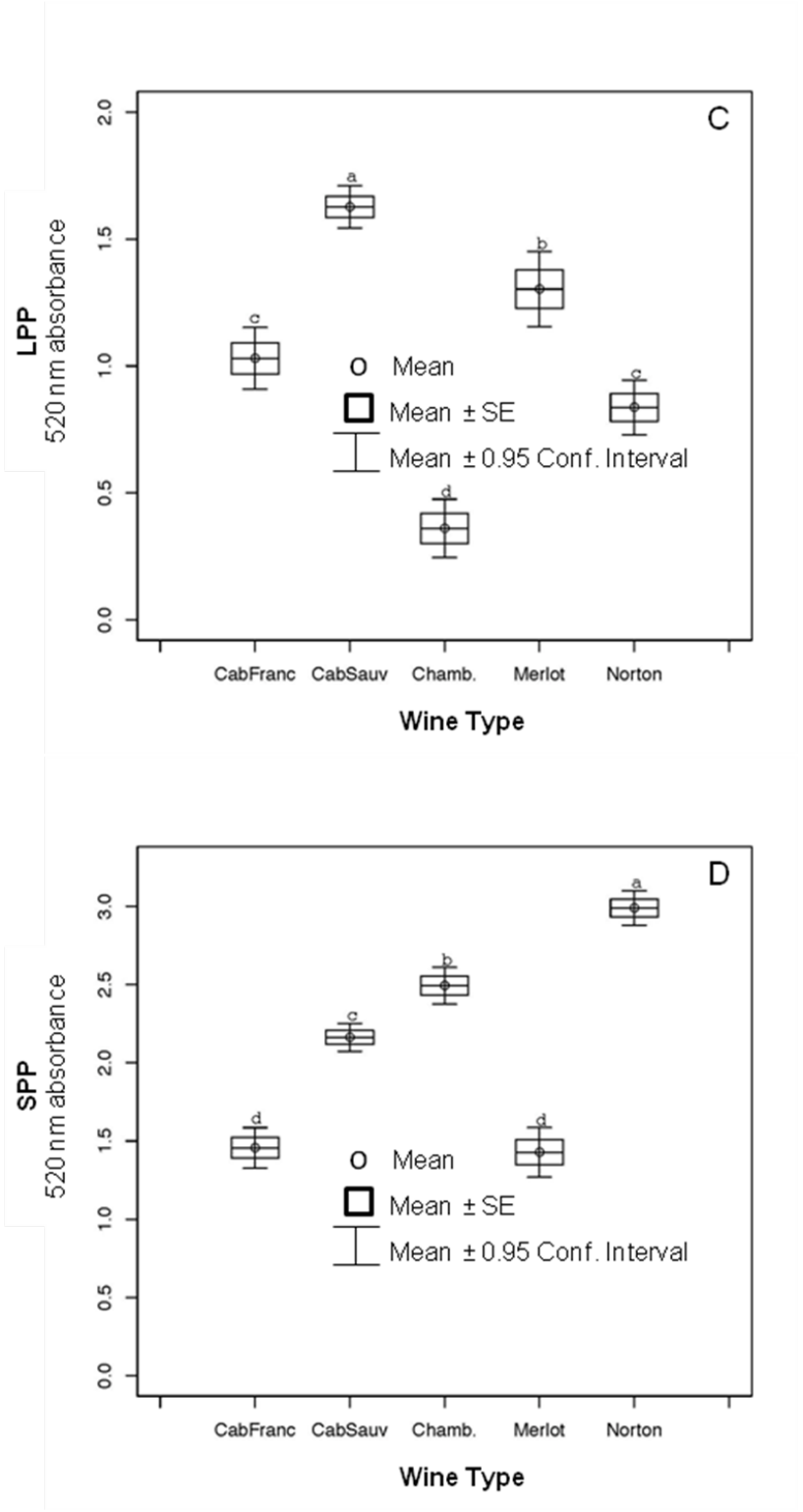

C
LPP
520 nm absorbance
0.0
0.5
1.0
1.5
2.0
a
b
c
c
d
Mean
Mean ± SE
Mean ± 0.95 Conf. Interval
CabFranc
CabSauv
Chamb.
Merlot
Norton
Wine Type
D
SPP
520 nm absorbance
0.0
0.5
1.0
1.5
2.0
2.5
3.0
a
b
c
d
d
Mean
Mean ± SE
Mean ± 0.95 Conf. Interval
CabFranc
CabSauv
Chamb.
Merlot
Norton
Wine Type

Figure A.3.1

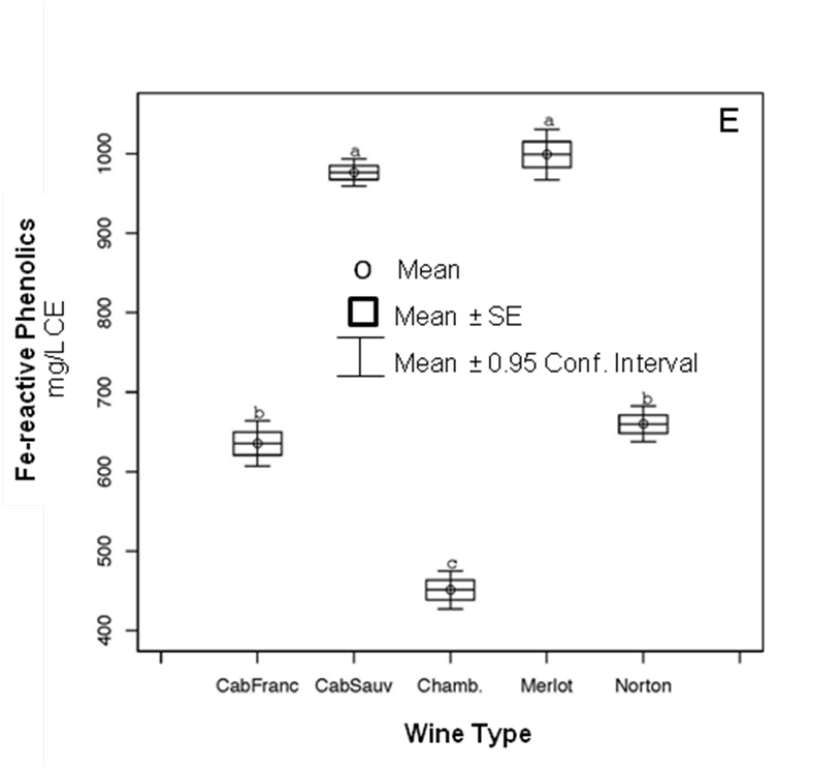


Figure A.3.1 Content of phenolic compounds according to wine type (**A**), tannin content in mg/L catechin equivalents (CE) of Cabernet Franc (CabFranc, n=10), Cabernet Sauvignon (CabSauv, n=28), Chambourcin (Chamb., n=14), Merlot (n=8), and Norton wines (n=16) (**B**), anthocyanin content of the wines (**C**), absorbance due to LPP at 520 nm of the wines (**D**), absorbance due to SPP at 520 nm of the wines (**E**), Fe-reactive phenolic content in mg/L CE of the wines. Mean values appear as a horizontal line are surrounded by a box signifying the SE and bars outside of the box represent the 95% confidence interval. The letters associated with the means indicate significant difference at $p \leq 0.05$ using Tukey's HSD (Honestly Significant Difference) test.

Appendix A.4: Cultivar Study

Table A.4.1. Cultivar berry mass, phenolic and anthocyanin content

Collection days after flowering		berry mass[a] (g FW)		phenolic content[a] (mg chlorogenic acid /100 g FW)		anthocyanin content[a] (mg malvidin-3-glucoside/100 g FW)	
2009	2010	2009	2010	2009	2010	2009	2010
			Cabernet Franc				
0	0	0.013 ± NA[b]	0.015 ± 0.000	873 ± NA	1398 ± 227	26 ± NA	7 ± 4
14	14	0.046 ± 0.000	0.311 ± 0.027	753 ± 75	244 ± 33	NA < 5	NA < 5
28	28	0.633 ± 0.050	0.894 ± 0.039	426 ± 46	567 ± 21	NA < 5	NA < 5
42	42	0.685 ± 0.081	1.072 ± 0.022	163 ± 19	356 ± 27	NA < 5	NA < 5
56	56	0.834 ± 0.013	1.271 ± 0.048	277 ± 42	654 ± 70	NA < 5	5 ± 4
70	70	1.306 ± 0.033	1.608 ± 0.029	292 ± 88	342 ± 25	35 ± 14	18 ± 4
84	84	1.452 ± 0.024	1.847 ± 0.097	107 ± 26	261 ± 29	44 ± 7	25 ± 3
99	98	1.419 ± 0.095	1.670 ± 0.005	153 ± 8	233 ± 1	69 ± 8	40 ± 3
113	112	1.331 ± 0.068	1.776 ± 0.099	149 ± 6	245 ± 17	47 ± 11	34 ± 6
122		1.340 ± 0.164		177 ± 44		72 ± 27	
			Cabernet Sauvignon				
0	0	0.014 ± NA	0.013 ± NA	1088 ± NA	1043 ± 153	31 ± NA	8 ± 3
14	14	0.051 ± 0.000	0.188 ± 0.000	781 ± 28	428 ± 116	6 ± 1	NA < 5
28	28	0.485 ± 0.018	0.709 ± 0.018	722 ± 304	508 ± 30	NA < 5	NA < 5
42	42	0.791 ± 0.024	0.930 ± 0.024	268 ± 13	247 ± 10	NA < 5	NA < 5
56	56	0.929 ± 0.119	1.063 ± 0.119	259 ± 24	466 ± 33	10 ± 1	NA < 5
70	70	1.305 ± 0.112	1.398 ± 0.112	212 ± 11	312 ± 20	45 ± 8	NA < 5
85	84	1.503 ± 0.043	1.351 ± 0.043	94 ± 21	267 ± 9	49 ± 5	8 ± 5
100	98	1.588 ± 0.088	1.461 ± 0.088	148 ± 28	222 ± 24	71 ± 10	12 ± 7
112	112	1.453 ± 0.126	1.400 ± 0.126	142 ± 23	250 ± 17	61 ± 13	22 ± 12
120	119	1.431 ± 0.039	1.437 ± 0.039	154 ± 16	259 ± 41	53 ± 6	23 ± 13
			Chambourcin				
0	0	0.010 ± NA	0.017 ± 0.000	942 ± NA	2007 ± 192	27 ± NA	22 ± 9
14	14	0.036 ± 0.000	0.579 ± 0.023	1021 ± 35	288 ± 76	5 ± 3	NA < 5
28	28	0.803 ± 0.072	1.293 ± 0.069	326 ± 36	554 ± 98	NA < 5	NA < 5
42	42	1.082 ± 0.072	1.608 ± 0.029	261 ± 28	267 ± 13	NA < 5	NA < 5
56	56	1.212 ± 0.123	1.847 ± 0.097	320 ± 2	270 ± 12	NA < 5	NA < 5
71	70	1.969 ± 0.110	2.436 ± 0.064	113 ± 12	155 ± 7	24 ± 7	39 ± 5
84	84	2.198 ± 0.055	2.436 ± 0.064	115 ± 30	176 ± 16	98 ± 17	84 ± 15
98	98	2.150 ± 0.089	2.253 ± 0.055	132 ± 50	255 ± 2	108 ± 43	153 ± 1
112	109	2.198 ± 0.055	2.198 ± 0.055	218 ± 37	380 ± 122	147 ± 27	204 ± 12
126		2.587 ± 0.140		244 ± 12		163 ± 16	
			Norton				
0	0	0.018 ± NA	0.014 ± 0.000	403 ± NA	676 ± 55	17 ± NA	45 ± 7
14	14	0.079 ± 0.000	0.262 ± 0.019	528 ± 51	260 ± 53	NA < 5	64 ± 3
28	28	0.512 ± 0.015	0.866 ± 0.008	818 ± 242	406 ± 17	11 ± 1	NA < 5
42	42	0.618 ± 0.042	0.884 ± 0.030	196 ± 38	276 ± 17	NA < 5	NA < 5
56	56	0.805 ± 0.027	1.072 ± 0.022	265 ± 8	506 ± 10	NA < 5	NA < 5
70	70	1.100 ± 0.035	1.225 ± 0.074	142 ± 7	307 ± 6	57 ± 8	10 ± 1
83	84	1.271 ± 0.048	1.242 ± 0.073	137 ± 28	220 ± 11	108 ± 18	59 ± 7
98	98	1.190 ± 0.059	1.306 ± 0.033	334 ± 15	244 ± 11	256 ± 13	102 ± 9
110	112	1.201 ± 0.028	1.387 ± 0.041	458 ± 3	388 ± 44	276 ± 16	185 ± 9
118		1.233 ± 0.017		416 ± 28		259 ± 26	

Table A.4.1 (Continued). Cultivar berry mass, phenolic and anthocyanin content

[a] The mean value ± the standard error. [b] NA represents no standard error determined due to only one replicate utilized, NA also represents that no analysis was done at the collection time when only NA is present for that particuar sampling date, and NA < 5 is used when the mean of anthocyanin was less than 5 mg/100 g FW. In all cases except when only one replicate was utilized, as denoted by the NA in place of the standard error, the replicates were n=3.

Table A.4.2. Cultivar berry tartaric acid and malic acid content

collection weeks after flowering		tartaric acid content[a] (mg tartaric acid /berry)		malic acid content[a] (mg malic acid/berry)	
2009	2010	2009	2010	2009	2010
			Cabernet Franc		
0	0	0.29 ± NA	0.33 ± 0.02	0.14 ± NA	0.08 ± 0.00
2	2	0.71 ± 0.03	3.05 ± 0.12	0.26 ± 0.03	2.20 ± 0.23
4	4	5.28 ± 0.75	6.56 ± 0.05	8.04 ± 0.73	10.74 ± 0.25
6	6	8.55 ± 1.08	8.85 ± 0.47	18.18 ± 2.16	19.03 ± 1.39
8	8	6.42 ± 0.49	9.42 ± 0.47	16.45 ± 0.74	20.21 ± 0.87
10	10	9.16 ± 0.56	8.80 ± 2.58	16.21 ± 1.27	8.79 ± 1.70
12	12	7.99 ± 1.10	4.38 ± 0.23	4.32 ± 0.68	3.17 ± 0.45
14	14	7.65 ± 0.40	9.31 ± 1.21	4.14 ± 0.62	3.03 ± 0.59
16	16	9.63 ± 1.72	10.74 ± 0.16	2.69 ± 0.35	3.57 ± 0.92
18		8.00 ± 0.66		4.35 ± 0.51	
			Cabernet Sauvignon		
0	0	0.21 ± NA	0.26 ± 0.01	0.04 ± NA	0.09 ± 0.00
2	2	0.86 ± 0.03	2.30 ± 0.09	0.30 ± 0.06	1.26 ± 0.24
4	4	6.44 ± 0.24	6.90 ± 0.36	8.35 ± 0.07	8.64 ± 0.44
6	6	8.18 ± 0.25	7.39 ± 0.30	14.61 ± 1.04	16.22 ± 0.19
8	8	9.11 ± 0.96	8.06 ± 0.54	18.63 ± 2.74	21.35 ± 2.23
10	10	9.01 ± 2.65	9.79 ± 3.10	14.85 ± 3.36	15.00 ± 3.75
12	12	8.65 ± 2.30	5.51 ± 0.82	7.73 ± 3.46	5.39 ± 0.91
14	14	9.96 ± 1.06	9.08 ± 1.32	6.64 ± 1.64	5.85 ± 0.55
16	16	11.04 ± 0.74	9.38 ± 1.61	5.59 ± 0.79	5.23 ± 0.66
18	17	11.04 ± 0.79	11.86 ± 1.44	5.31 ± 0.80	5.31 ± 0.74

Table A.4.2 (Continued). Cultivar berry tartaric acid and malic acid content

collection weeks after flowering		tartaric acid content[a] (mg tartaric acid /berry)		malic acid content[a] (mg malic acid/berry)	
2009	2010	2009	2010	2009	2010
			Chambourcin		
0	0	0.21 ± NA	0.35 ± 0.04	0.02 ± NA	0.03 ± 0.01
2	2	0.80 ± 0.01	6.54 ± 1.17	0.08 ± 0.01	3.37 ± 0.44
4	4	9.19 ± 1.14	14.92 ± 0.45	8.73 ± 0.76	19.03 ± 0.11
6	6	12.55 ± 1.99	22.35 ± 5.10	15.46 ± 3.15	29.33 ± 1.82
8	8	15.53 ± 1.77	15.92 ± 0.27	28.14 ± 3.14	20.86 ± 1.41
10	10	15.47 ± 1.03	10.17 ± 0.30	22.06 ± 1.21	7.30 ± 1.84
12	12	15.81 ± 1.39	9.99 ± 0.60	15.78 ± 0.74	4.54 ± 0.82
14	14	17.24 ± 4.60	8.53 ± 2.25	15.10 ± 6.94	4.06 ± 0.38
16	16	23.41 ± 6.08	19.97 ± 1.60	14.65 ± 1.50	6.61 ± 0.46
18		19.43 ± 0.81		11.34 ± 1.84	
			Norton		
0	0	0.71 ± NA	0.30 ± 0.04	0.21 ± NA	0.05 ± 0.00
2	2	1.74 ± 0.52	2.80 ± 0.08	0.90 ± 0.35	1.57 ± 0.07
4	4	10.35 ± 3.40	7.44 ± 0.17	14.81 ± 5.69	9.48 ± 0.54
6	6	11.51 ± 1.60	8.89 ± 0.66	22.82 ± 3.05	15.64 ± 1.39
8	8	12.66 ± 1.78	10.03 ± 0.34	33.86 ± 4.26	21.92 ± 1.39
10	10	14.26 ± 3.25	7.11 ± 0.41	28.05 ± 9.91	11.90 ± 0.77
12	12	10.34 ± 1.34	6.98 ± 0.22	13.75 ± 3.44	5.98 ± 0.24
14	14	7.92 ± 0.85	6.50 ± 0.18	5.22 ± 1.85	5.17 ± 0.31
16	16	8.09 ± 1.18	4.77 ± 0.13	6.43 ± 0.76	2.62 ± 0.41
18		8.15 ± 1.08		3.28 ± 0.57	

[a] The mean value ± the standard error. [b] NA represents no standard error determined due to only one replicate utilized. In all cases except when only one replicate was utilized, as denoted by the NA in place of the standard error, the replicates were n=3.

Table A.4.3. Cultivar berry mass, oxalic acid and citric acid content

collection weeks after flowering		berry mass[a] (g FW)		oxalic acid content[a] (mg oxalic acid/berry)		citric acid content[a] (mg citric acid/berry)	
2009	2010	2009	2010	2009	2010	2009	2010
				Cabernet Franc			
0	0	0.013 ± NA[b]	0.030 ± 0.000	0.10 ± NA	0.22 ± 0.01	NA < 0.005	NA < 0.005
2	2	0.046 ± 0.000	0.302 ± 0.016	0.14 ± 0.02	0.30 ± 0.13	NA < 0.005	NA < 0.005
4	4	0.623 ± 0.062	0.764 ± 0.008	0.44 ± 0.04	0.38 ± 0.11	0.15 ± 0.02	0.22 ± 0.01
6	6	0.799 ± 0.057	0.954 ± 0.102	0.68 ± 0.20	0.42 ± 0.11	0.25 ± 0.03	0.41 ± 0.04
8	8	0.868 ± 0.054	1.154 ± 0.062	0.51 ± 0.05	0.71 ± 0.10	0.24 ± 0.00	0.48 ± 0.02
10	10	1.599 ± 0.081	1.681 ± 0.156	1.42 ± 0.17	1.02 ± 0.36	0.22 ± 0.11	0.54 ± 0.15
12	12	1.349 ± 0.161	1.752 ± 0.011	0.74 ± 0.09	0.44 ± 0.07	0.08 ± 0.08	0.30 ± 0.19
14	14	1.354 ± 0.121	1.790 ± 0.090	0.92 ± 0.14	1.22 ± 0.16	0.24 ± 0.03	0.42 ± 0.05
16	16	1.393 ± 0.240	1.643 ± 0.121	0.89 ± 0.19	1.50 ± 0.05	0.29 ± 0.04	0.54 ± 0.11
18		1.426 ± 0.216		0.89 ± 0.13		0.35 ± 0.07	
				Cabernet Sauvignon			
0	0	0.014 ± NA	0.067 ± 0.000	0.08 ± NA	0.14 ± 0.01	NA < 0.005	NA < 0.005
2	2	0.051 ± 0.000	0.200 ± 0.000	0.12 ± 0.02	0.20 ± 0.02	NA < 0.005	NA < 0.005
4	4	0.549 ± 0.037	0.785 ± 0.030	0.65 ± 0.18	0.60 ± 0.08	0.12 ± 0.01	0.23 ± 0.00
6	6	0.788 ± 0.052	0.904 ± 0.020	1.23 ± 0.24	0.46 ± 0.06	0.24 ± 0.02	0.46 ± 0.02
8	8	1.042 ± 0.129	1.161 ± 0.114	0.98 ± 0.18	0.71 ± 0.16	0.30 ± 0.07	0.67 ± 0.09
10	10	1.549 ± 0.158	1.348 ± 0.045	0.83 ± 0.18	1.02 ± 0.37	0.39 ± 0.09	0.83 ± 0.21
12	12	1.474 ± 0.087	1.627 ± 0.050	0.71 ± 0.20	0.46 ± 0.11	0.29 ± 0.17	0.39 ± 0.08
14	14	1.600 ± 0.046	1.634 ± 0.120	1.01 ± 0.13	0.83 ± 0.15	0.44 ± 0.06	0.69 ± 0.12
16	16	1.508 ± 0.082	1.459 ± 0.066	1.04 ± 0.15	1.05 ± 0.19	0.47 ± 0.01	0.79 ± 0.08
18	17	1.722 ± 0.033	1.466 ± 0.126	1.06 ± 0.27	1.41 ± 0.13	0.52 ± 0.06	0.96 ± 0.17
				Chambourcin			
0	0	0.010 ± NA	0.033 ± 0.001	0.04 ± NA	0.05 ± 0.01	NA < 0.005	NA < 0.005
2	2	0.036 ± 0.000	0.494 ± 0.077	0.08 ± 0.00	0.57 ± 0.12	NA < 0.005	NA < 0.005
4	4	0.802 ± 0.069	1.374 ± 0.101	0.75 ± 0.15	0.64 ± 0.14	0.12 ± 0.01	0.35 ± 0.01
6	6	1.196 ± 0.161	1.656 ± 0.041	1.12 ± 0.27	1.16 ± 0.14	0.16 ± 0.08	0.67 ± 0.07
8	8	1.352 ± 0.162	1.808 ± 0.069	0.92 ± 0.20	1.07 ± 0.13	0.41 ± 0.03	0.63 ± 0.05
10	10	1.961 ± 0.177	2.154 ± 0.181	0.85 ± 0.28	1.52 ± 0.22	0.39 ± 0.03	1.05 ± 0.20
12	12	2.379 ± 0.075	2.495 ± 0.169	1.37 ± 0.24	1.27 ± 0.22	0.74 ± 0.09	0.66 ± 0.05
14	14	2.008 ± 0.078	2.551 ± 0.009	2.37 ± 1.14	1.09 ± 0.36	0.71 ± 0.26	0.90 ± 0.26
16	16	2.388 ± 0.145	2.436 ± 0.052	2.86 ± 0.40	3.10 ± 0.17	1.12 ± 0.28	1.82 ± 0.05
18		2.561 ± 0.107		2.65 ± 0.27		1.13 ± 0.08	
				Norton			
0	0	0.018 ± NA	0.030 ± 0.000	0.16 ± NA	0.08 ± 0.01	0.01 ± NA	NA < 0.005
2	2	0.079 ± 0.000	0.302 ± 0.016	0.14 ± 0.05	0.29 ± 0.01	0.02 ± 0.01	NA < 0.005
4	4	0.539 ± 0.033	0.764 ± 0.008	0.59 ± 0.23	0.57 ± 0.02	0.22 ± 0.08	0.22 ± 0.01
6	6	0.644 ± 0.027	0.954 ± 0.102	0.62 ± 0.14	0.95 ± 0.07	0.36 ± 0.04	0.46 ± 0.02
8	8	0.883 ± 0.049	1.154 ± 0.062	1.17 ± 0.13	1.21 ± 0.11	0.68 ± 0.10	0.83 ± 0.03
10	10	1.241 ± 0.073	1.681 ± 0.156	1.80 ± 0.75	0.87 ± 0.06	0.82 ± 0.33	0.76 ± 0.01
12	12	1.240 ± 0.135	1.752 ± 0.011	1.23 ± 0.58	1.01 ± 0.17	0.54 ± 0.15	0.85 ± 0.12
14	14	1.025 ± 0.064	1.790 ± 0.090	0.99 ± 0.10	0.76 ± 0.06	0.42 ± 0.03	0.71 ± 0.07
16	16	1.233 ± 0.066	1.644 ± 0.121	1.24 ± 0.10	0.71 ± 0.03	0.50 ± 0.03	0.59 ± 0.11
18		1.066 ± 0.108		1.11 ± 0.12		0.39 ± 0.03	

Table A.4.3 (Continued). Cultivar berry mass, oxalic acid and citric acid content

[a] The mean value ± the standard error. [b] NA represents no standard error determined due to only one replicate utilized, and NA < 0.005 is for when the citric acid mean was less than 0.005 mg/berry. In all cases except when only one replicate was utilized, as denoted by the NA in place of standard error, the replicates were n=3.

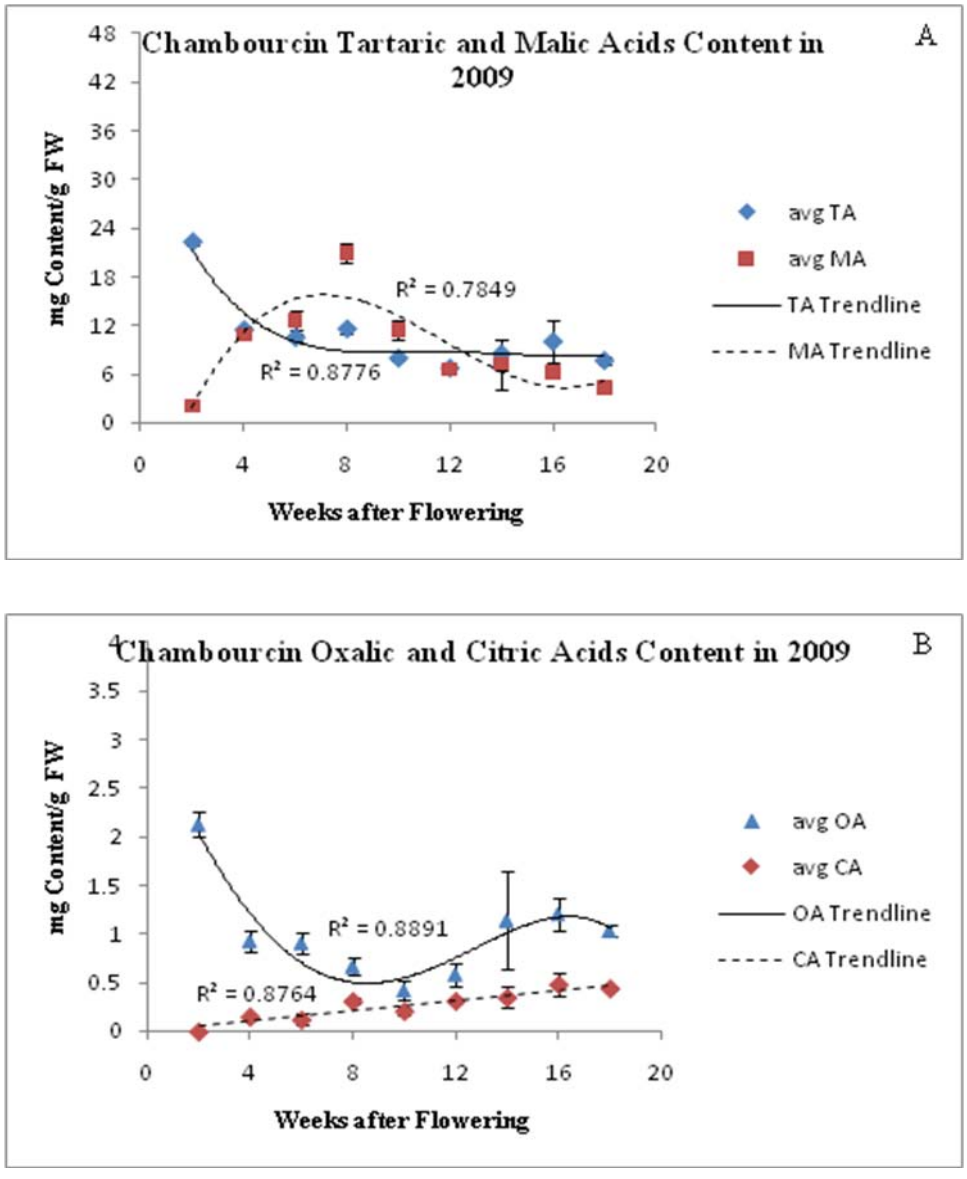
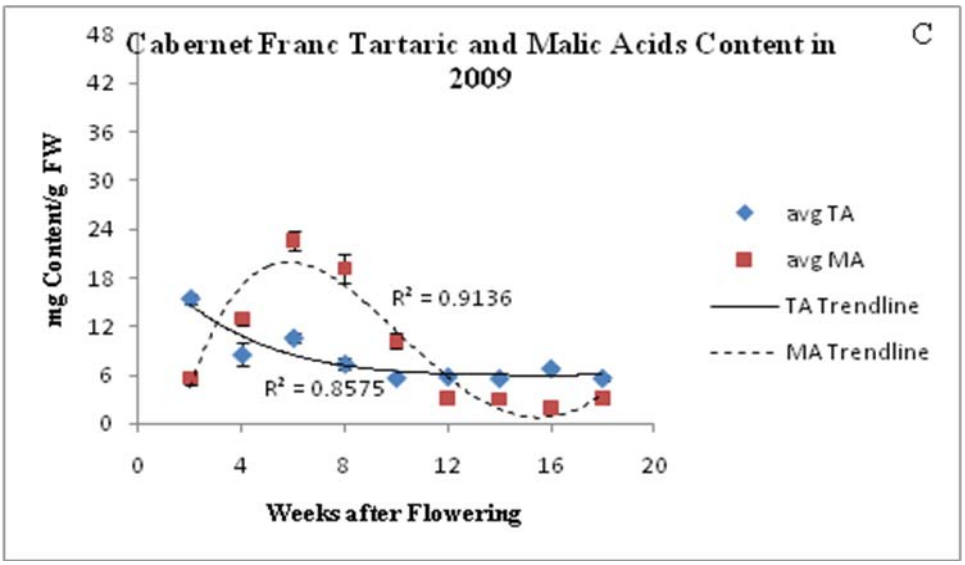


Figure A.4.1


C
Cabernet Franc Tartaric and Malic Acids Content in 2009
mg Content/g FW
Weeks after Flowering
avg TA
avg MA
TA Trendline
MA Trendline
R² = 0.9136
R² = 0.8575

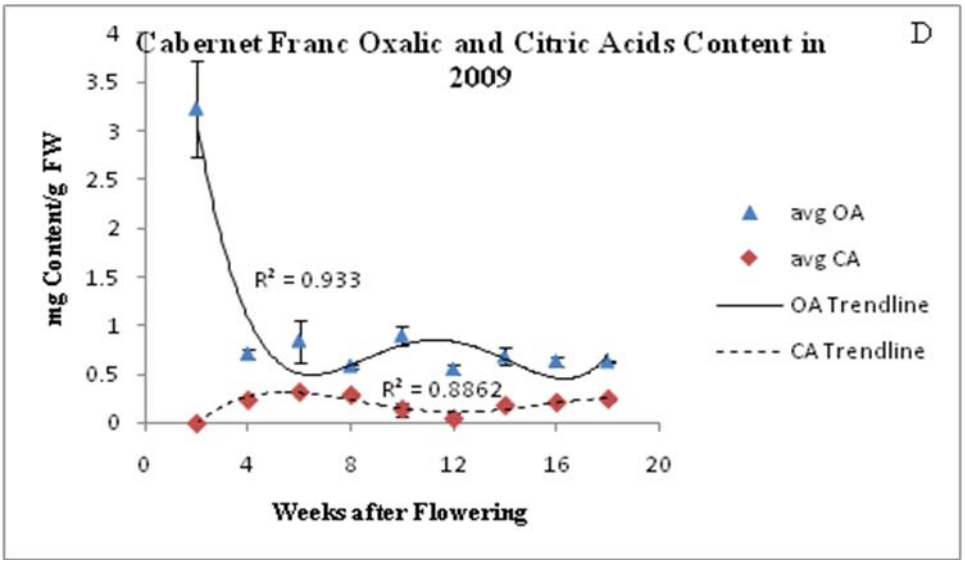
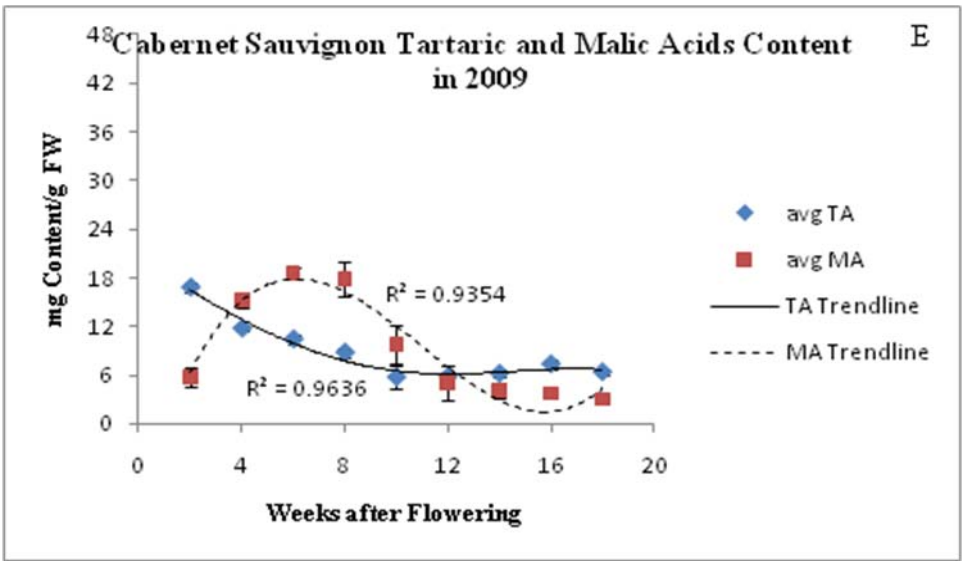

D
Cabernet Franc Oxalic and Citric Acids Content in 2009
mg Content/g FW
Weeks after Flowering
avg OA
avg CA
OA Trendline
CA Trendline
R² = 0.933
R² = 0.8862

Figure A.4.1


Cabernet Sauvignon Tartaric and Malic Acids Content in 2009
E
mg Content/g FW
Weeks after Flowering
avg TA
avg MA
TA Trendline
MA Trendline
R² = 0.9354
R² = 0.9636

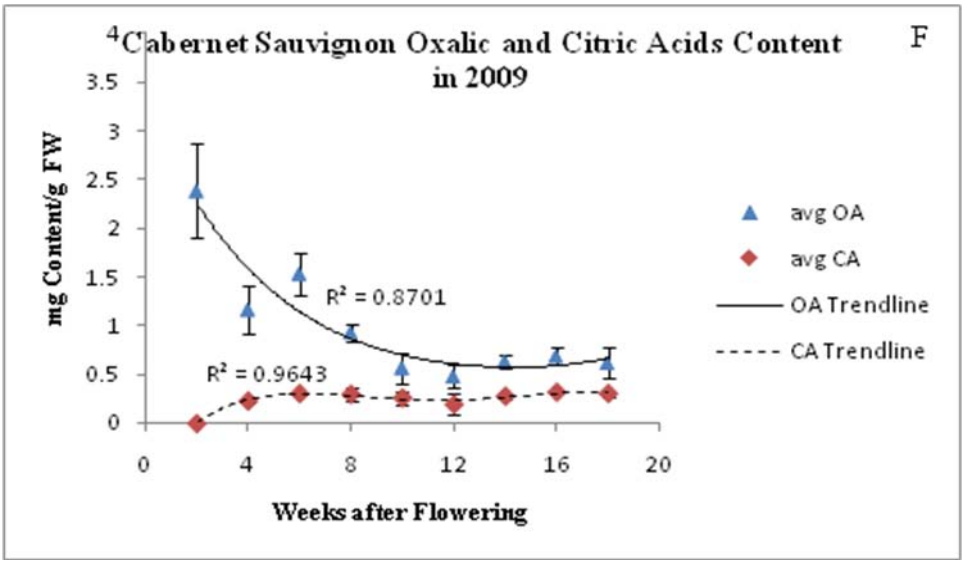
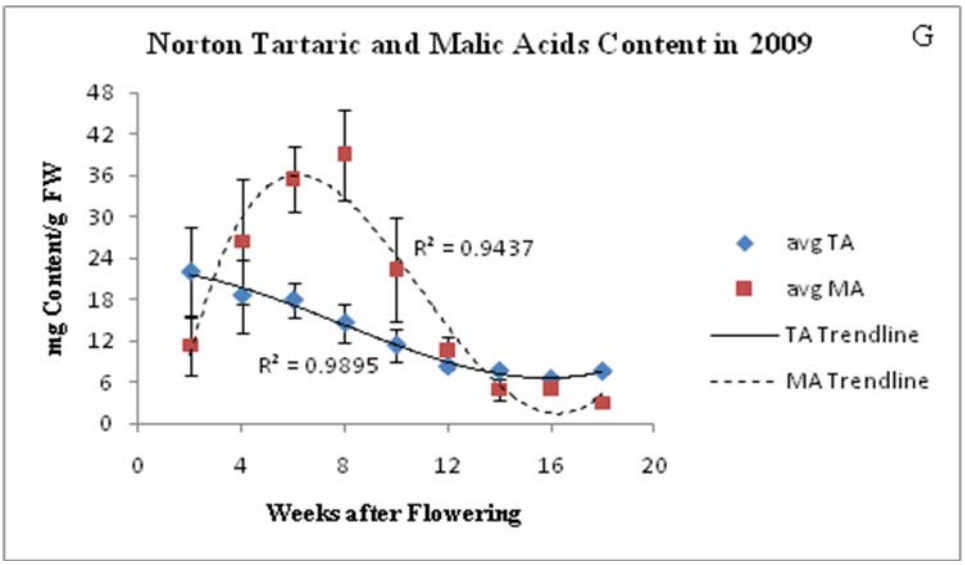

Cabernet Sauvignon Oxalic and Citric Acids Content in 2009
F
mg Content/g FW
Weeks after Flowering
avg OA
avg CA
OA Trendline
CA Trendline
R² = 0.8701
R² = 0.9643

Figure A.4.1



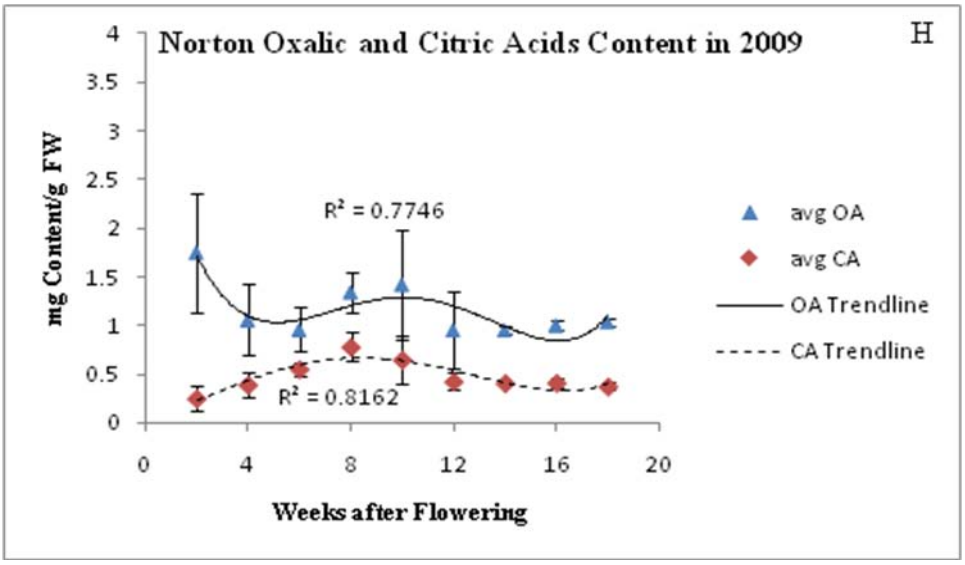
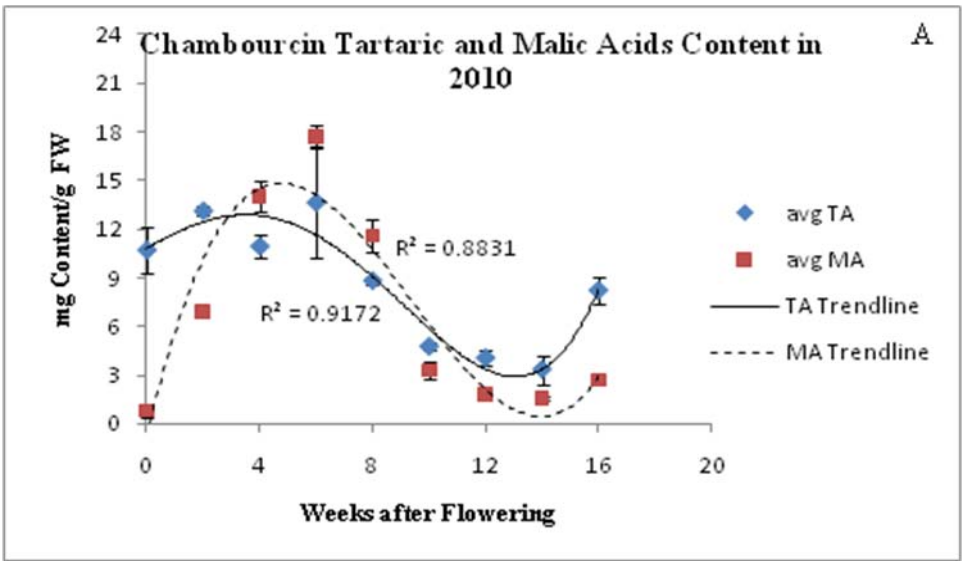


Figure A.4.1. Tartaric acid and malic acid content (**A, C, E, G**) for the grape cultivars Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton, respectfully and oxalic acid and citric acid content of Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton, (**B, D, F, H**). The collecting starting at two weeks after flowering on June 10th, June 15th, June 17th, and June 19th, 2009, respectfully, and all four cultivars were collected subsequently every two weeks until their harvest. Data points for Avg TA and MA, or OA and CA, represent mean contents and bars with caps represent standard errors.


A
Chambourcin Tartaric and Malic Acids Content in 2010
mg Content/g FW
Weeks after Flowering
R² = 0.8831
R² = 0.9172
avg TA
avg MA
TA Trendline
MA Trendline

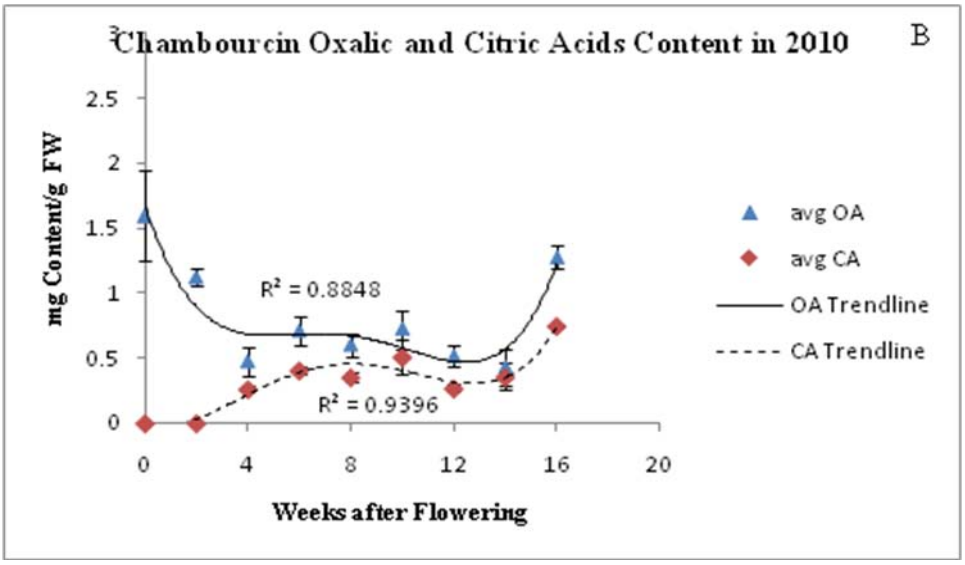
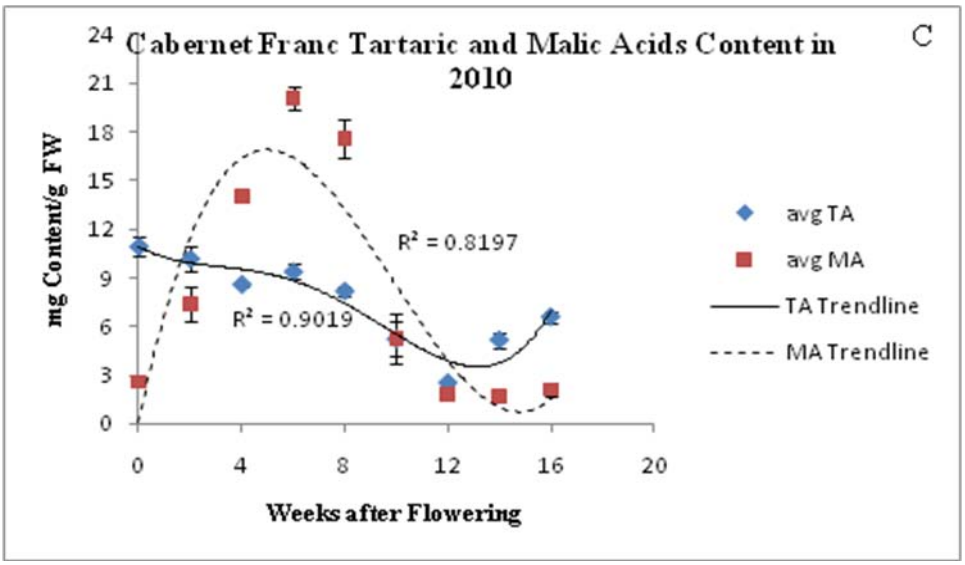

B
Chambourcin Oxalic and Citric Acids Content in 2010
mg Content/g FW
Weeks after Flowering
R² = 0.8848
R² = 0.9396
avg OA
avg CA
OA Trendline
CA Trendline

Figure A.4.2


C
Cabernet Franc Tartaric and Malic Acids Content in 2010
mg Content/g FW
Weeks after Flowering
R² = 0.8197
R² = 0.9019
avg TA
avg MA
TA Trendline
MA Trendline

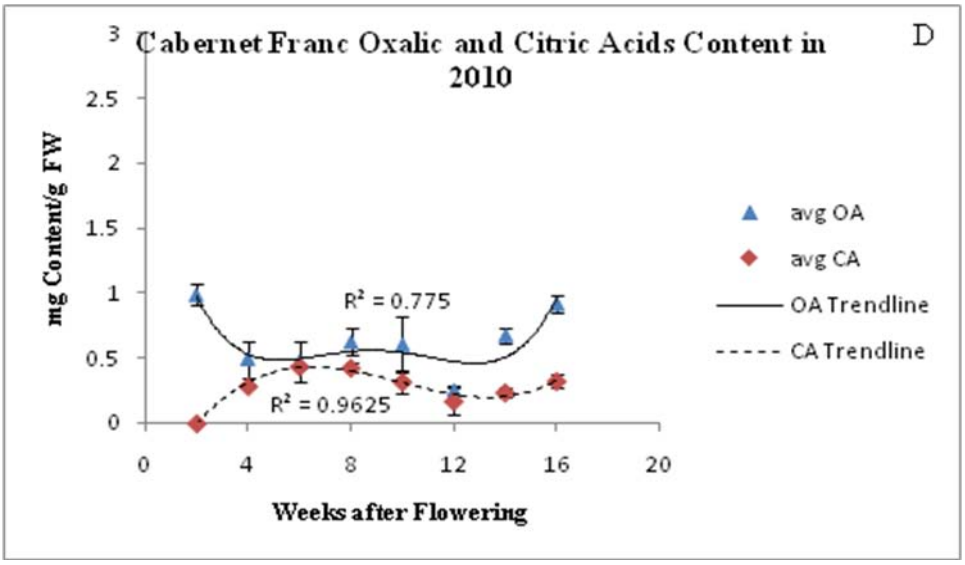
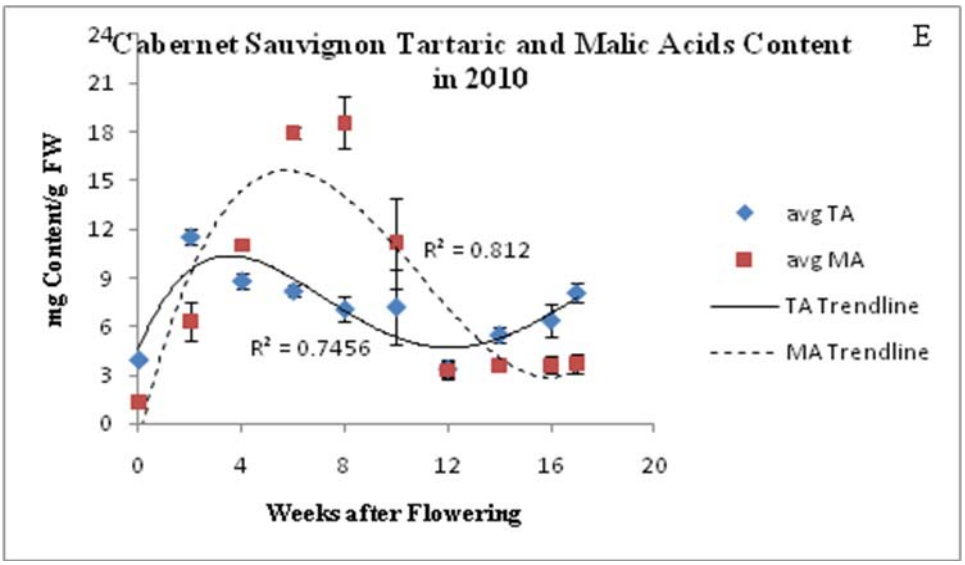

D
Cabernet Franc Oxalic and Citric Acids Content in 2010
mg Content/g FW
Weeks after Flowering
R² = 0.775
R² = 0.9625
avg OA
avg CA
OA Trendline
CA Trendline

Figure A.4.2


24Cabernet Sauvignon Tartaric and Malic Acids Content in 2010
E
mg Content/g FW
Weeks after Flowering
R² = 0.812
R² = 0.7456
avg TA
avg MA
TA Trendline
MA Trendline

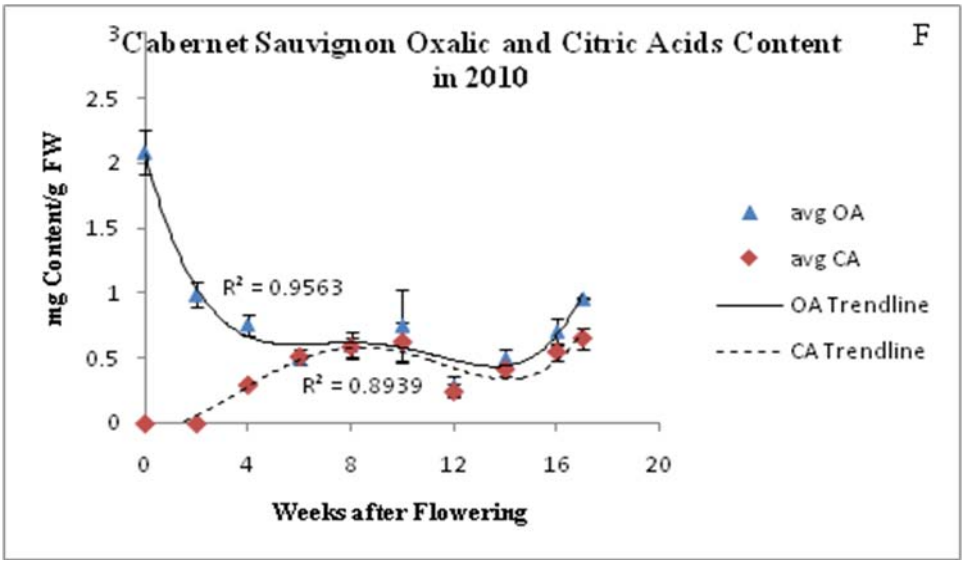
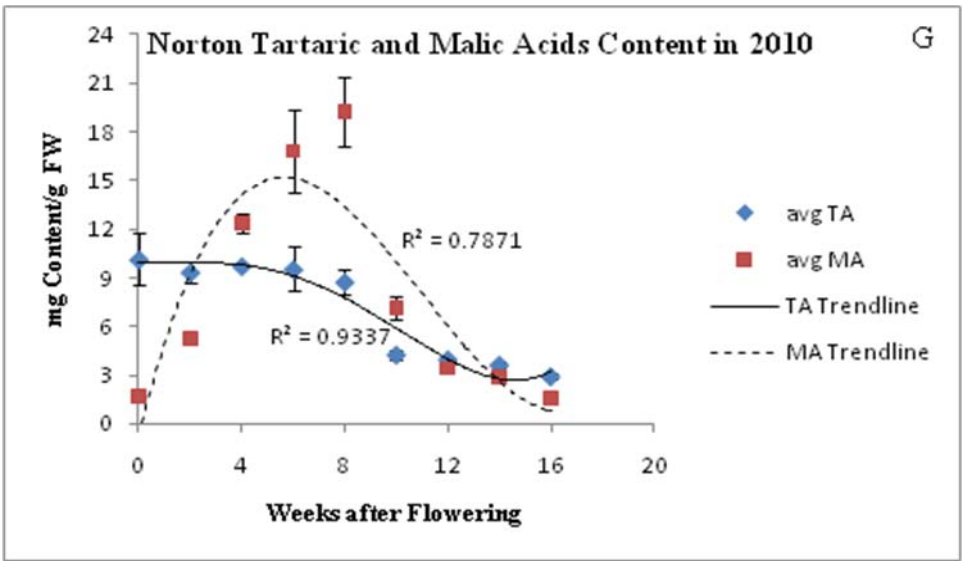

3Cabernet Sauvignon Oxalic and Citric Acids Content in 2010
F
mg Content/g FW
Weeks after Flowering
R² = 0.9563
R² = 0.8939
avg OA
avg CA
OA Trendline
CA Trendline

Figure A.4.2



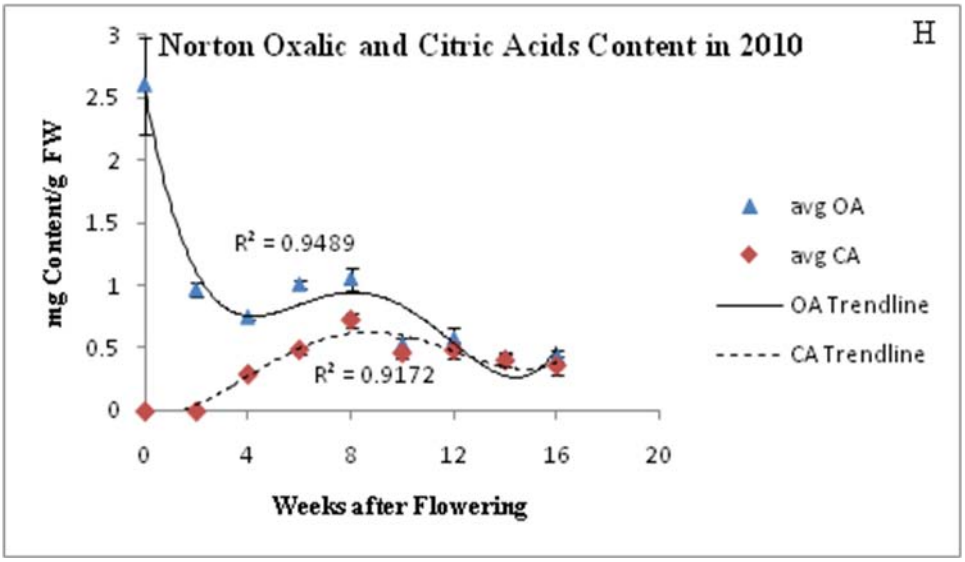


Figure A.4.2. Tartaric acid and malic acid content (**A, C, E, G**) for the grape cultivars Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton, respectfully and oxalic acid and citric acid content of Chambourcin, Cabernet Franc, Cabernet Sauvignon, and Norton, (**B, D, F, H**). The collecting starting at flowering on June 3rd, 2010, and all four cultivars were collected subsequently every two weeks until their harvest. Data points for Avg TA and MA, or OA and CA, represent mean contents and bars with caps represent standard errors.

Appendix A.5: Cabernet Franc Treatment Study

Table A.5.1. Spray treatment influence on Cabernet Franc phenolic content and anthocyanin content

collection date 2010	treatment	berry mass[a] (g FW)	phenolic content[a] (mg chlorogenic acid /100 g FW)	anthocyanin content[a] (mg malvidin-3-glucoside/100 g FW)
9/14/2010	control	1.809 ± 0.027 ns	195 ± 9 ns	44 ± 5 ns
9/14/2010	EtOH + Etp	1.783 ± 0.089 ns	200 ± 13 ns	45 ± 5 ns

[a] The mean value ± the standard error. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. The term ns was used because no treatment content was significantly different at the collection. Spray treatments were only applied to vines in the second year of study in 2010.

Table A.5.2. Cabernet Franc spray treatment berry mass, brix, and pH measurements

collection date 2010	treatment	berry mass[a] (g FW)	brix[a] (°Brix)	pH[a] (pH)
9/14/2010	control	1.809 ± 0.027 ns	21.6 ± 0.3 ns	3.52 ± 0.02 b
9/14/2010	EtOH + Etp	1.783 ± 0.089 ns	21.4 ± 0.3 ns	3.63 ± 0.02 a

[a] The mean value ± the standard error. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. In the case of significant difference in mean values, different letters represent that treatments are significantly different from one another. The ranking of treatments is in descending order starting with (a) representing the highest mean values, the term ns used if no treatment content was significantly different at the collection. Spray treatments were only applied to vines in the second year of study in 2010.

Table A.5.3. Spray treatment titratable acidity, berry tartaric acid and malic acid content

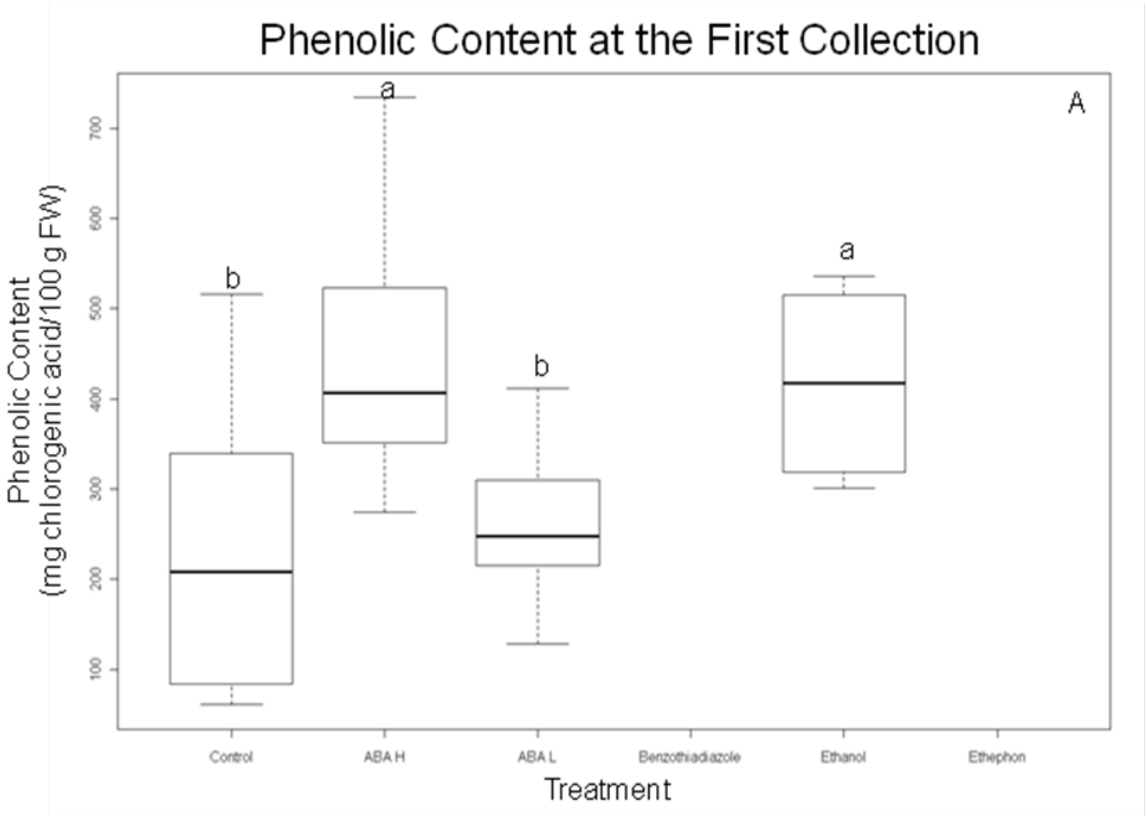
collection date 2010	treatment	titratable acidity[a] (g titratable acids/ L juice)	tartaric acid content[a] (mg tartaric acid /berry)	malic acid content[a] (mg malic acid/berry)
8/20	control	6.58 ± 0.52 ns	7.82 ± 0.72 ns	3.05 ± 0.27 ns
8/20	EtOH + Etp	6.44 ± 0.27 ns	6.64 ± 1.07 ns	3.40 ± 0.57 ns

[a] The mean value ± the standard error. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. The term ns was used because no treatment content was significantly different at the collection. Spray treatments were only applied the second year of study in 2010.

Table A.5.4. Spray treatment berry mass, berry oxalic acid and citric acid content

collection date 2010	treatment	berry mass[a] (g FW)	oxalic acid content[a] (mg oxalic acid/berry)	citric acid content[a] (mg citric acid/berry)
8/20	control	1.691 ± 0.038 ns	1.14 ± 0.11 ns	0.33 ± 0.04 ns
8/20	EtOH + Etp	1.662 ± 0.148 ns	0.99 ± 0.19 ns	0.27 ± 0.11 ns

[a] The mean value ± the standard error. The lowercase letters represent significant difference in mean values using Tukey's honestly significant difference test with $p \leq 0.05$. The term ns was used because no treatment content was significantly different at the collection. Spray treatments were only applied the second year of study in 2010.


Phenolic Content at the First Collection
A
a
b
b
a
Phenolic Content
(mg chlorogenic acid/100 g FW)
100
200
300
400
500
600
700
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

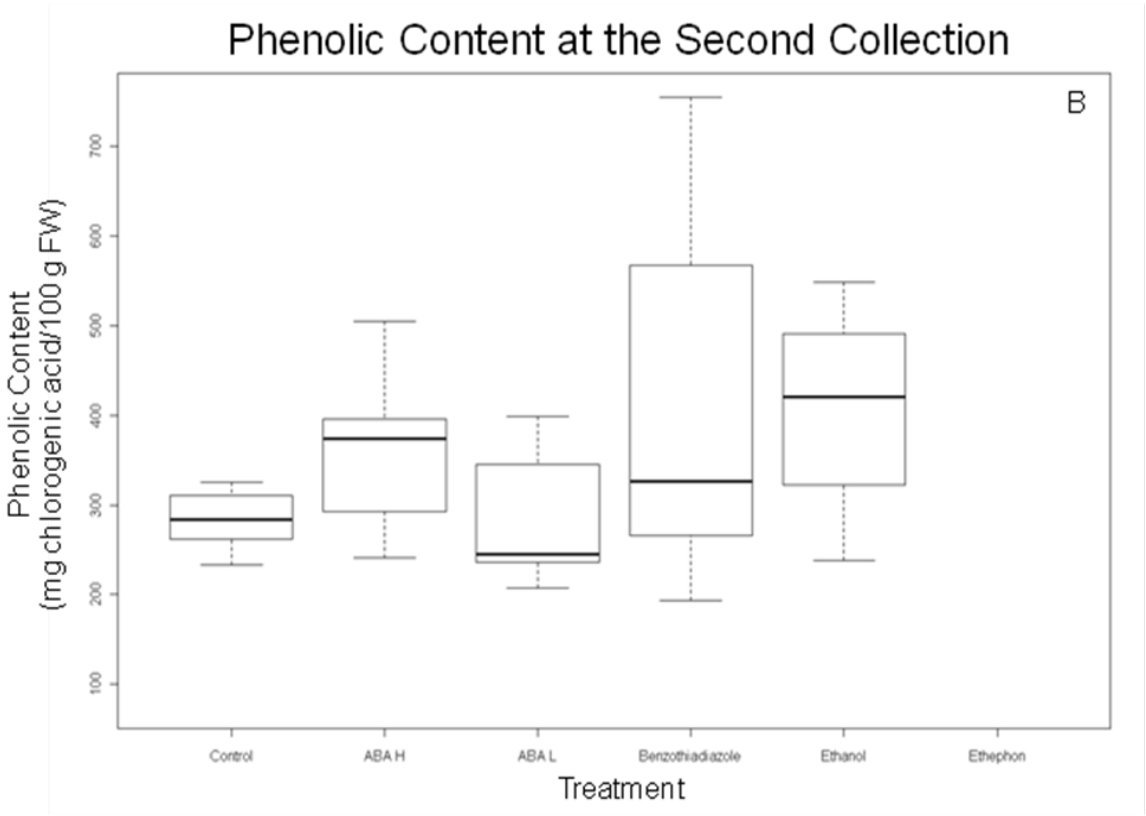
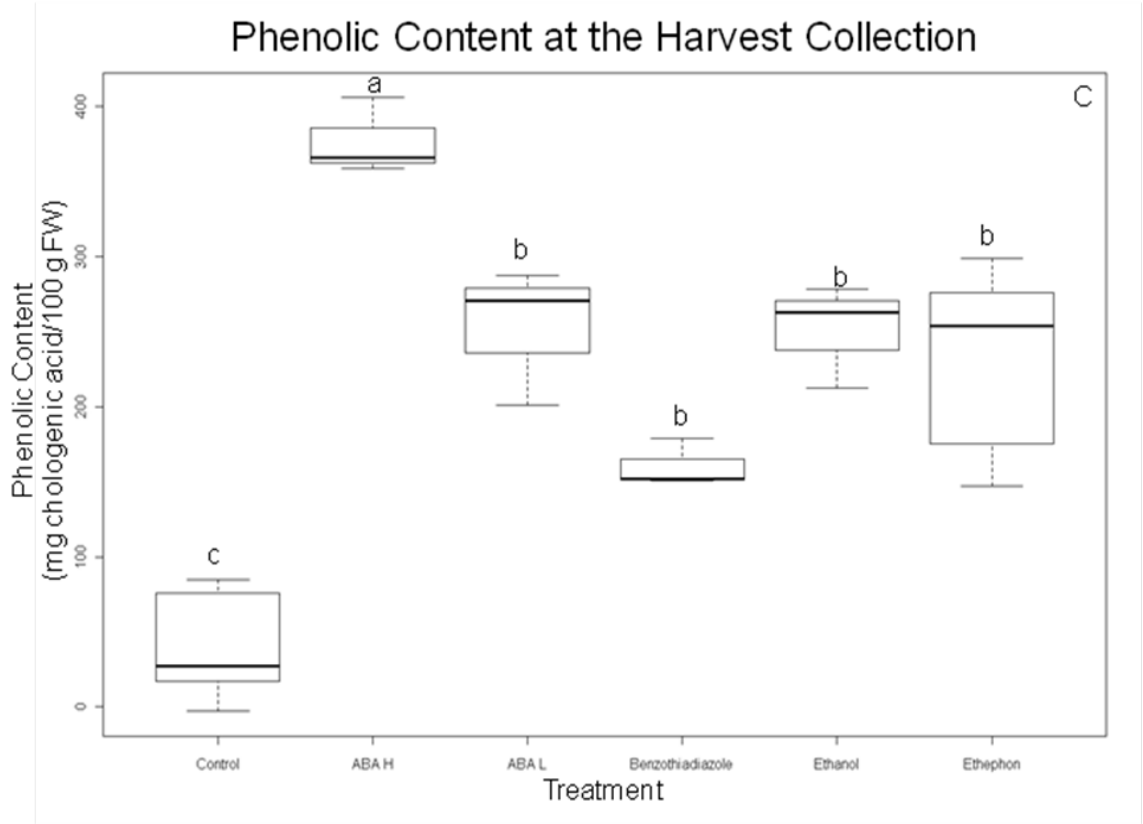

Phenolic Content at the Second Collection
B
Phenolic Content
(mg chlorogenic acid/100 g FW)
100
200
300
400
500
600
700
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

Figure A.5.1


Phenolic Content at the Harvest Collection
C
a
b
b
b
b
c
Phenolic Content
(mg chologenic acid/100 g FW)
0
100
200
300
400
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

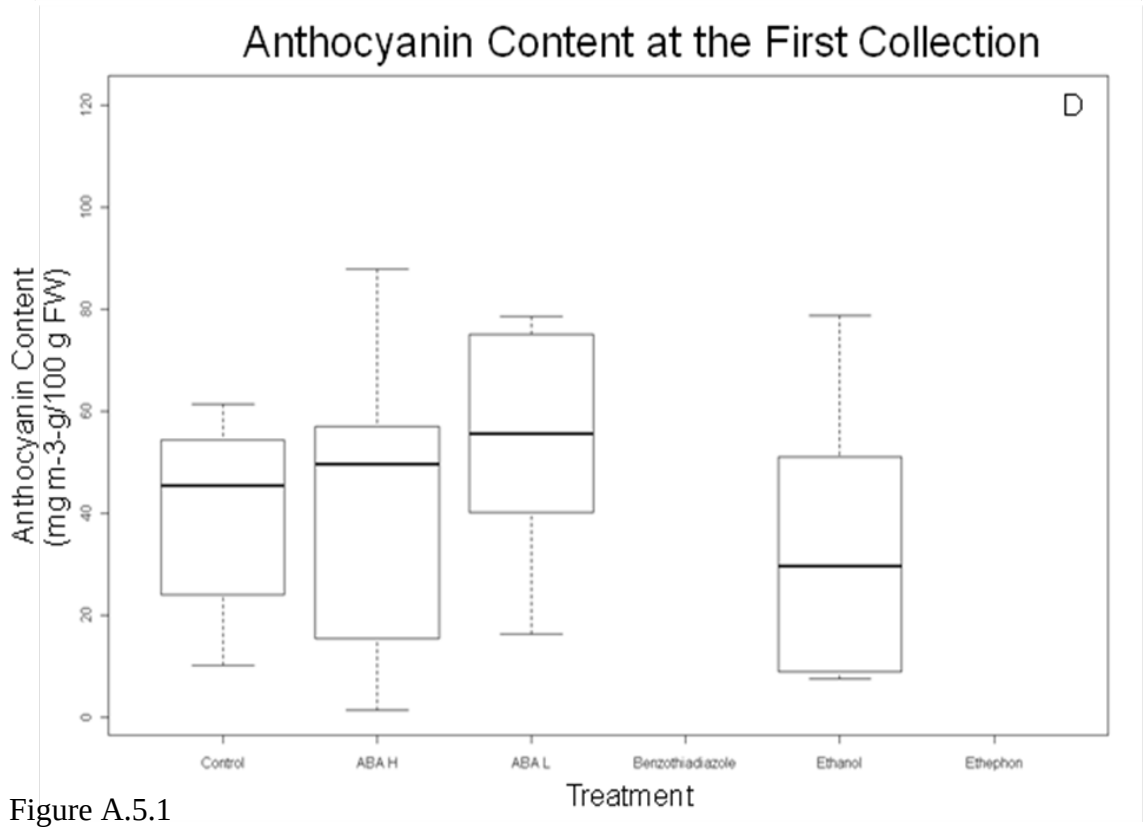
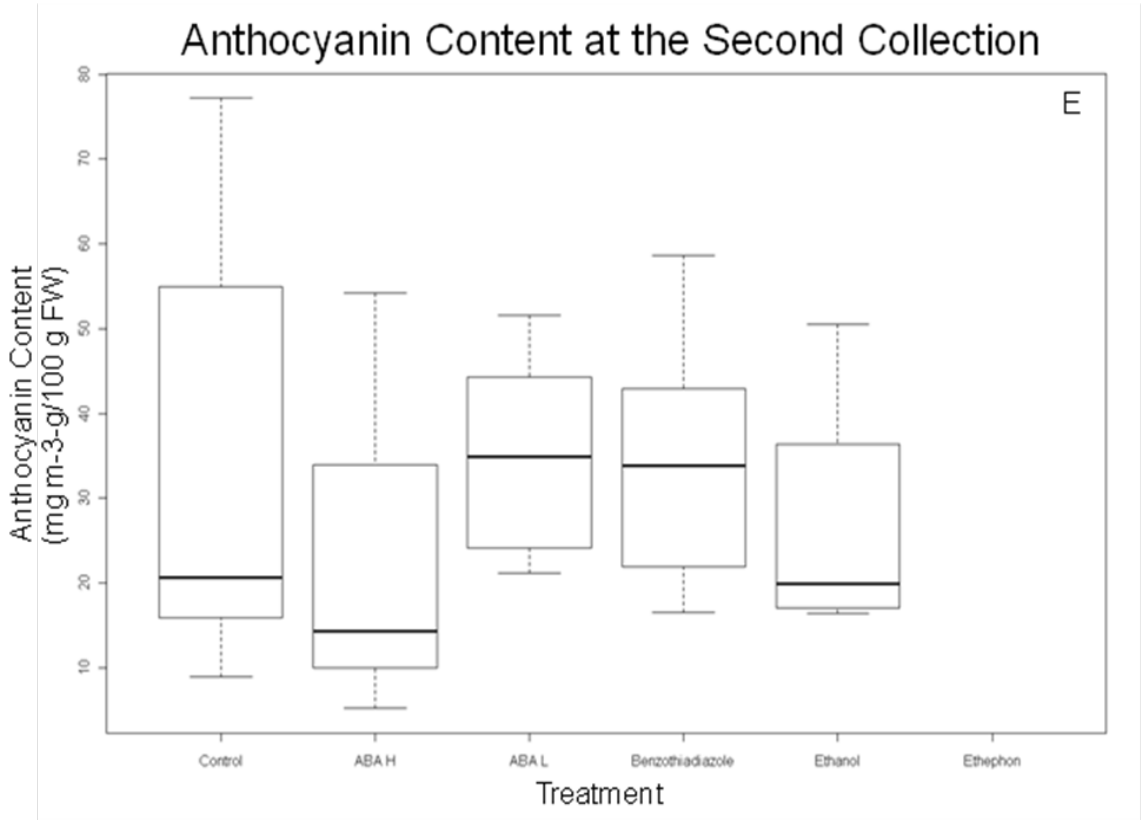

Anthocyanin Content at the First Collection
D
Anthocyanin Content
(mg m-3-g/100 g FW)
0
20
40
60
80
100
120
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

Figure A.5.1


Anthocyanin Content at the Second Collection
E
Anthocyanin Content
(mg m-3-g/100 g FW)
10
20
30
40
50
60
70
80
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

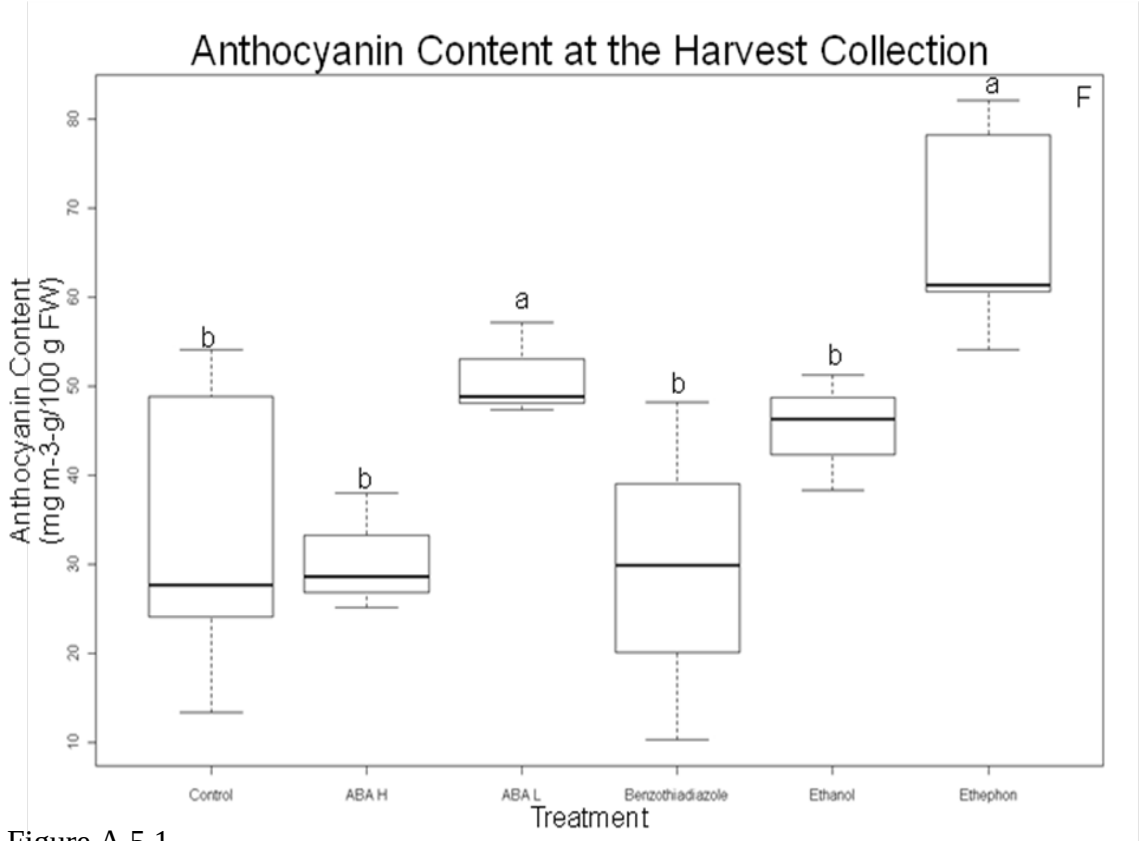
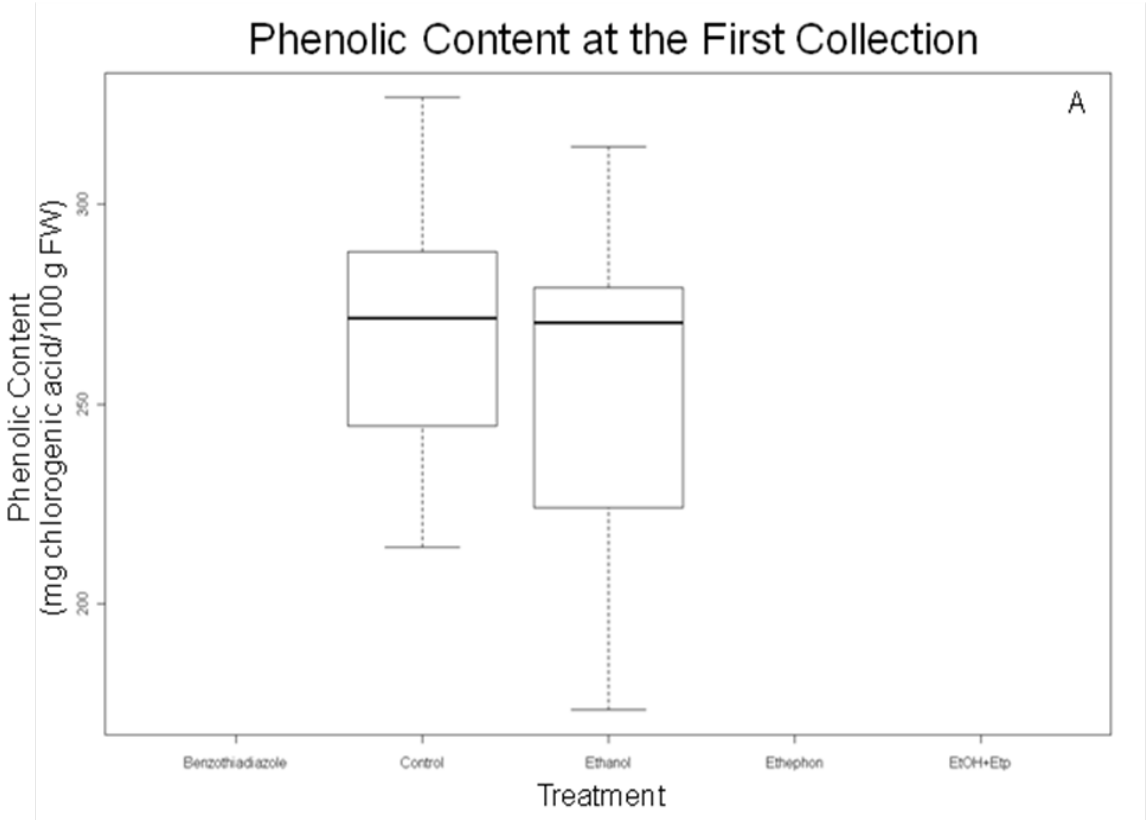

Anthocyanin Content at the Harvest Collection
F
Anthocyanin Content
(mg m-3-g/100 g FW)
10
20
30
40
50
60
70
80
b
b
a
b
b
a
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

Figure A.5.1

Figure A.5.1. Grape phenolic content of treatment Cabernet Franc grape clusters collected at August 24th, September 1st, and harvest on September 29th, 2009 with ABA high (ABA H), ABA low (ABA L), Benzothiadiazole, Control, Ethanol, and Ethephon treatments. (**A, B, C**), phenolic content of the treatments expressed as mg chlorogenic acid /100 g FW berries of the first collection, second collection, and harvest collection, respectfully (**D, E, F**), anthocyanin content of the treatments expressed as mg malvidin-3-glucoside (m-3-g)/100g FW berries of the first, second, and harvest collections, respectfully. For phenolic content and anthocyanin content, significant difference in the mean content, $p \leq 0.05$, using Tukey's HSD (Honestly Significant Difference) test was denoted using lowercase letters. For each treatment, the heavy horizontal black line represents mean content, the box contains the upper and lower quartiles, and the bars with caps represents either the minimum/maximum or 1.5 times the interquartile range, whichever is smaller.


Phenolic Content at the First Collection
A
Phenolic Content
(mg chlorogenic acid/100 g FW)
200
250
300
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

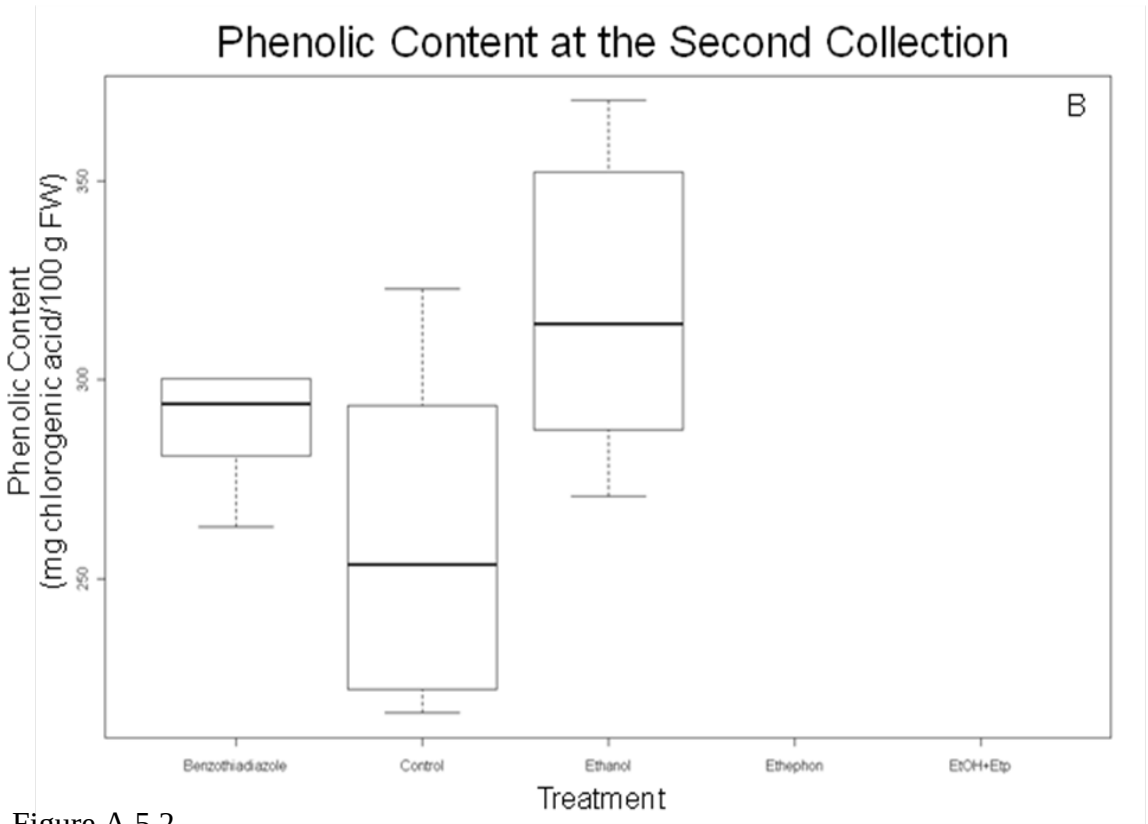
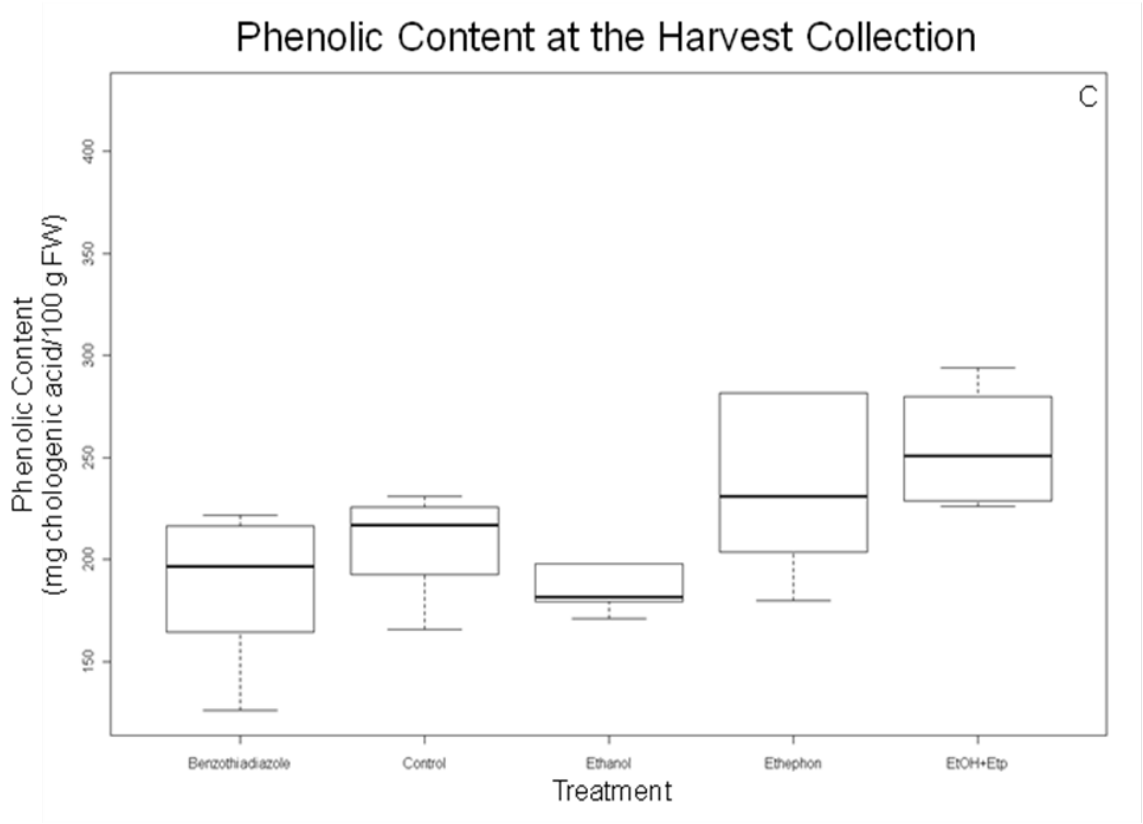

Phenolic Content at the Second Collection
B
Phenolic Content
(mg chlorogenic acid/100 g FW)
250
300
350
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

Figure A.5.2


Phenolic Content at the Harvest Collection
C
Phenolic Content
(mg chologenic acid/100 g FW)
150
200
250
300
350
400
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

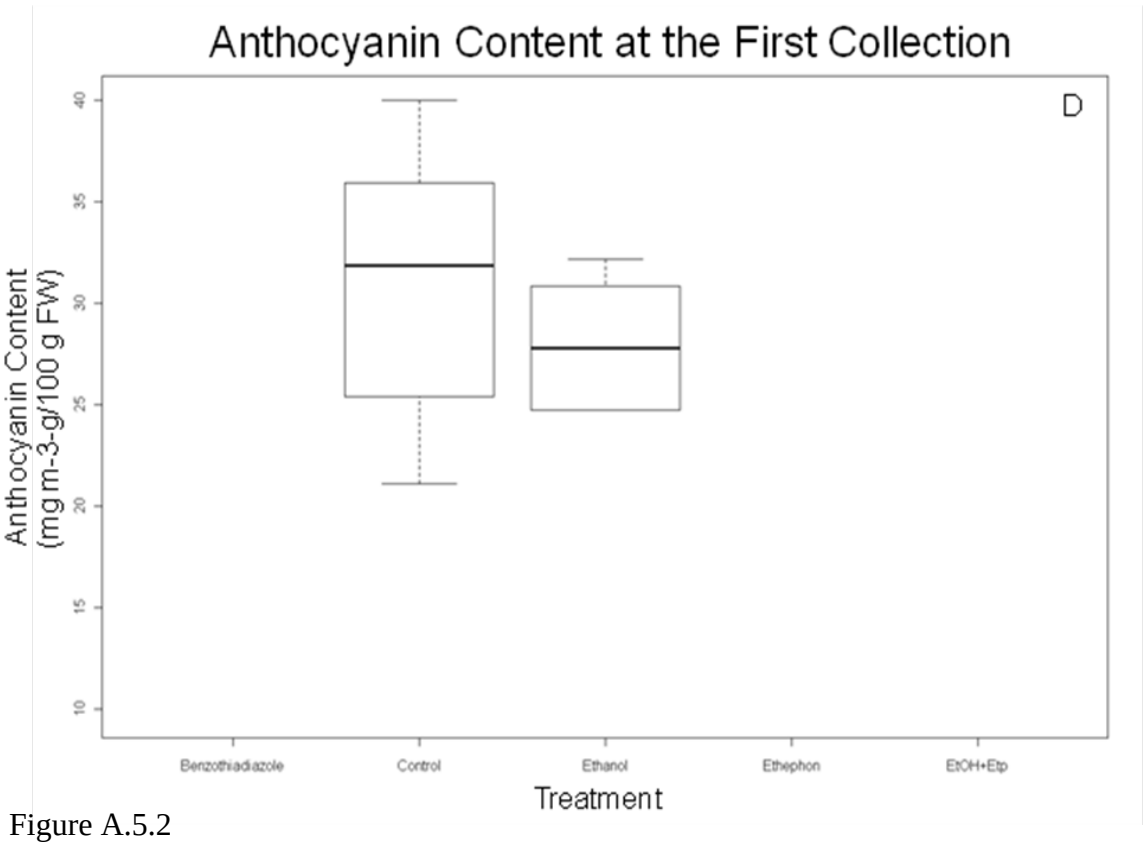
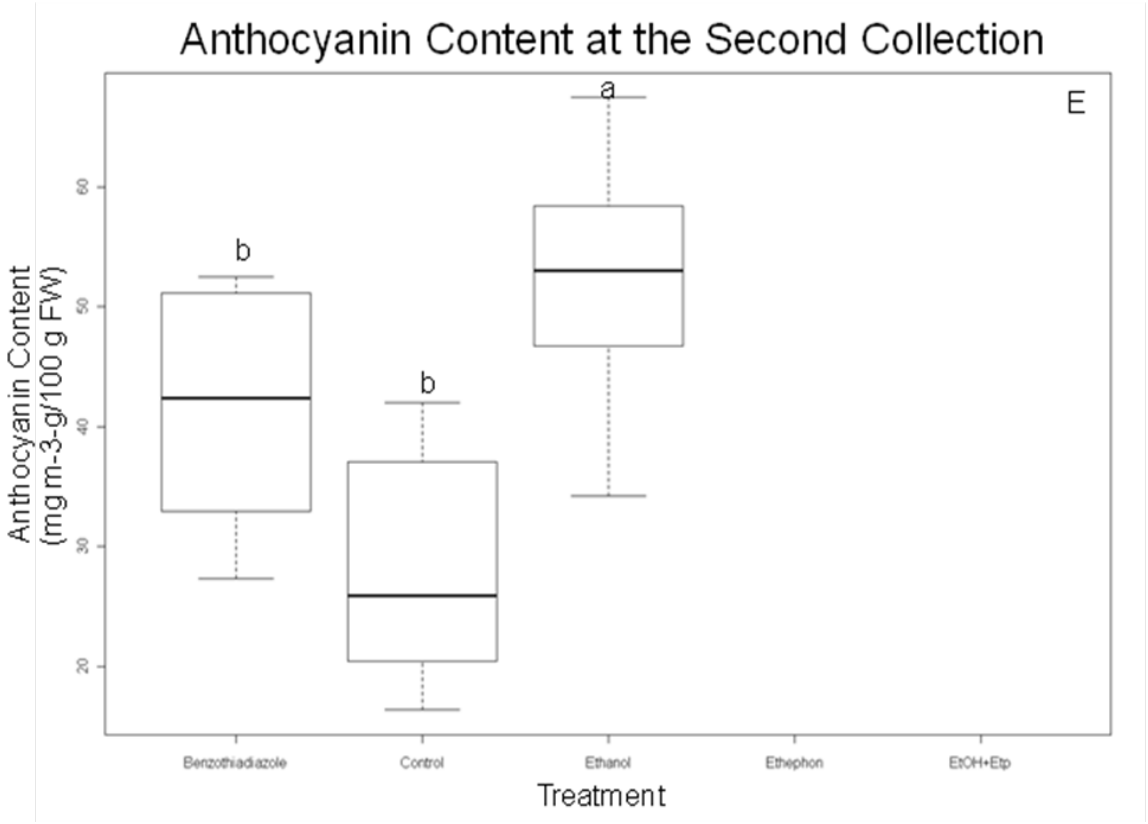

Anthocyanin Content at the First Collection
D
Anthocyanin Content
(mg m-3-g/100 g FW)
10
15
20
25
30
35
40
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

Figure A.5.2


Anthocyanin Content at the Second Collection
E
a
b
b
Anthocyanin Content
(mg m-3-g/100 g FW)
20
30
40
50
60
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

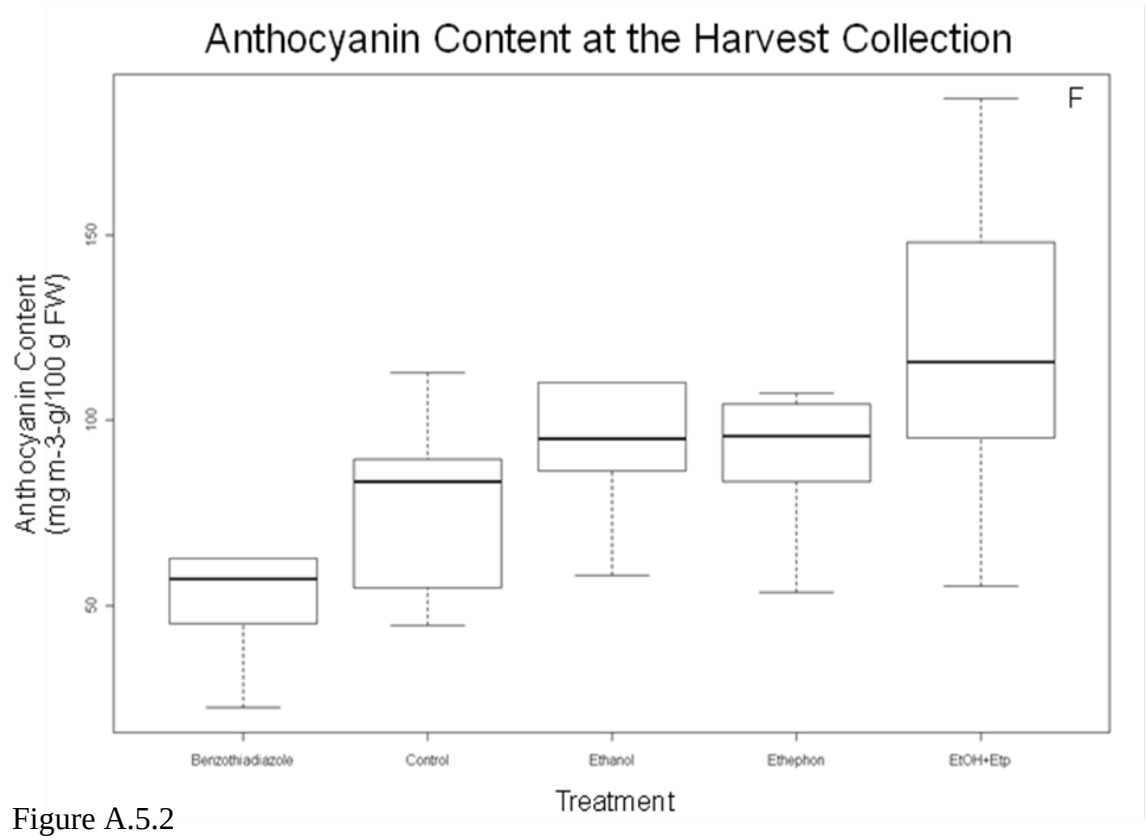
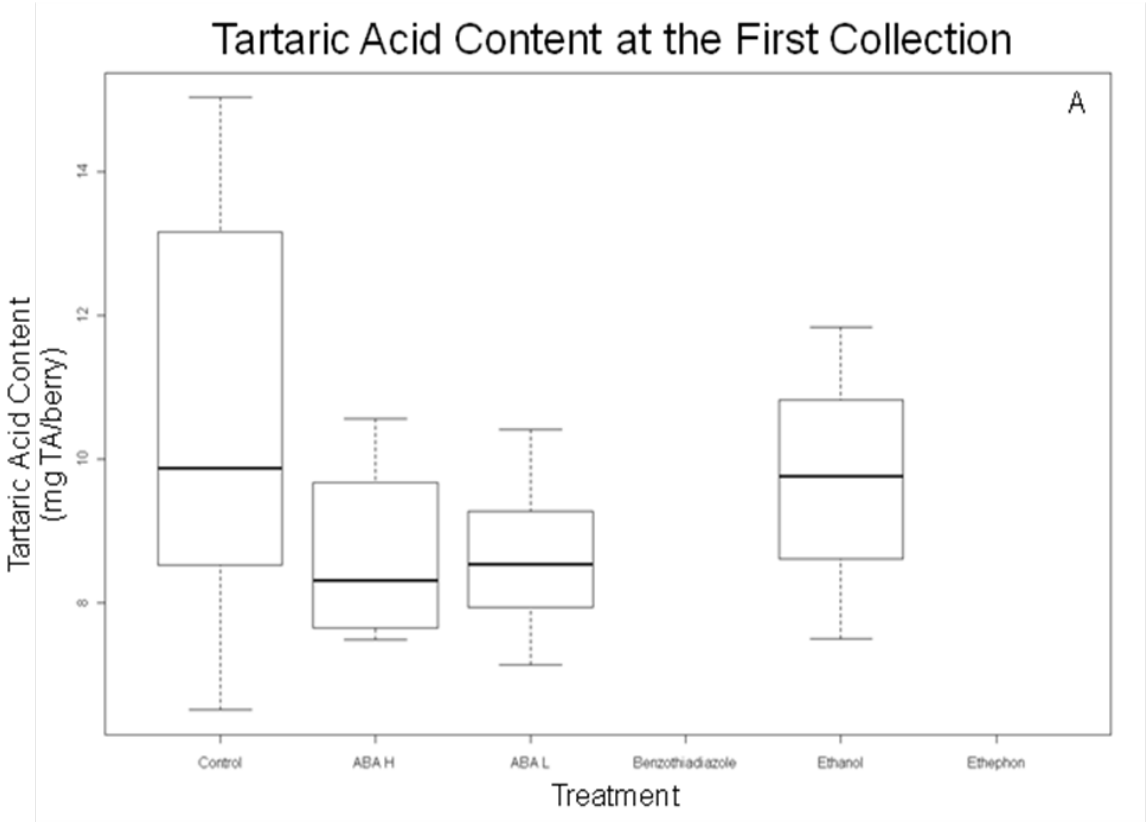

Anthocyanin Content at the Harvest Collection
F
Anthocyanin Content
(mg m-3-g/100 g FW)
50
100
150
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

Figure A.5.2

Figure A.5.2. Grape phenolic content of treatment Cabernet Franc grape clusters collected at August 20th, August 27th, and harvest on September 14th, 2010 with Benzothiadiazole, Control, Ethanol, Ethephon, and Ethanol with Ethephon (EtOH+Etp) treatments. (**A, B, C**), phenolic content of the treatments expressed as mg chlorogenic acid /100 g FW berries of the first collection, second collection, and harvest collection, respectfully (**D, E, F**), anthocyanin content of the treatments expressed as mg malvidin-3-glucoside (m-3-g)/100g FW berries of the first, second, and harvest collections, respectfully. For anthocyanin content, significant difference in the mean content, $p \leq 0.05$, using Tukey's HSD (Honestly Significant Difference) test was denoted using lowercase letters. For each treatment, the heavy horizontal black line represents mean content, the box contains the upper and lower quartiles, and the bars with caps represents either the minimum/maximum or 1.5 times the interquartile range, whichever is smaller.


Tartaric Acid Content at the First Collection
A
Tartaric Acid Content
(mg TA/berry)
8
10
12
14
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

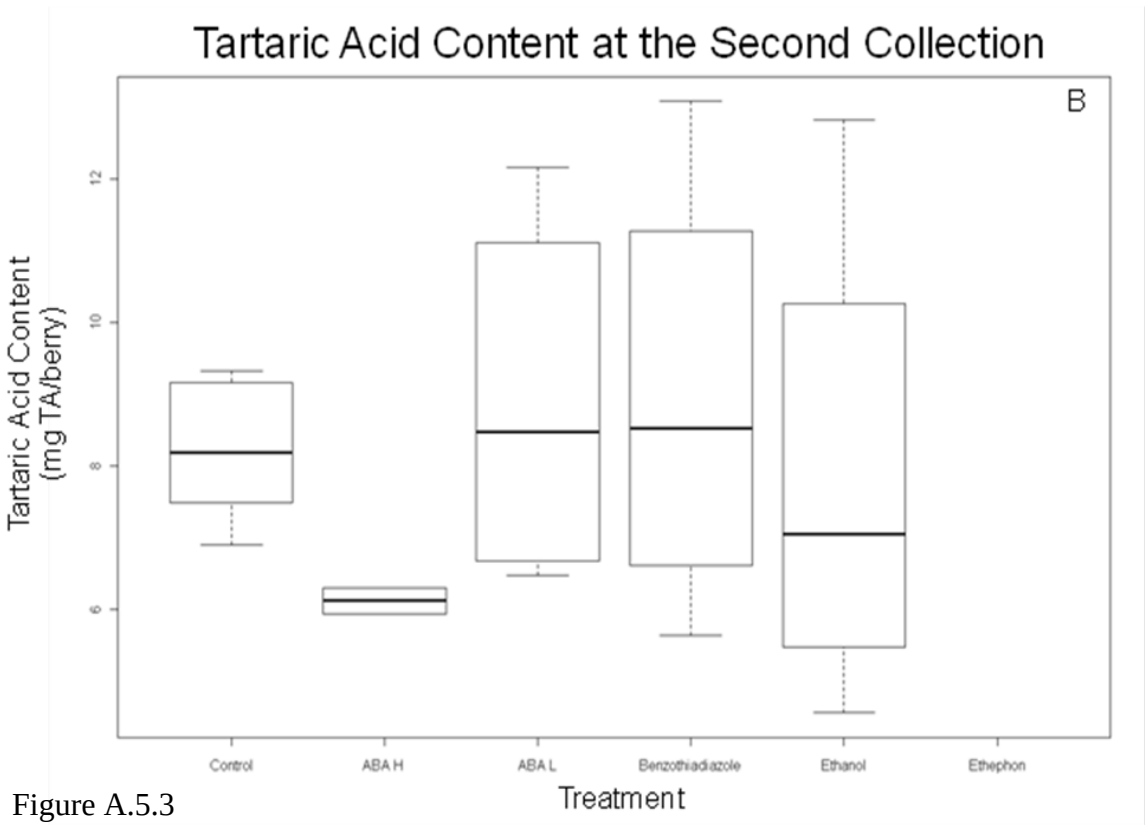
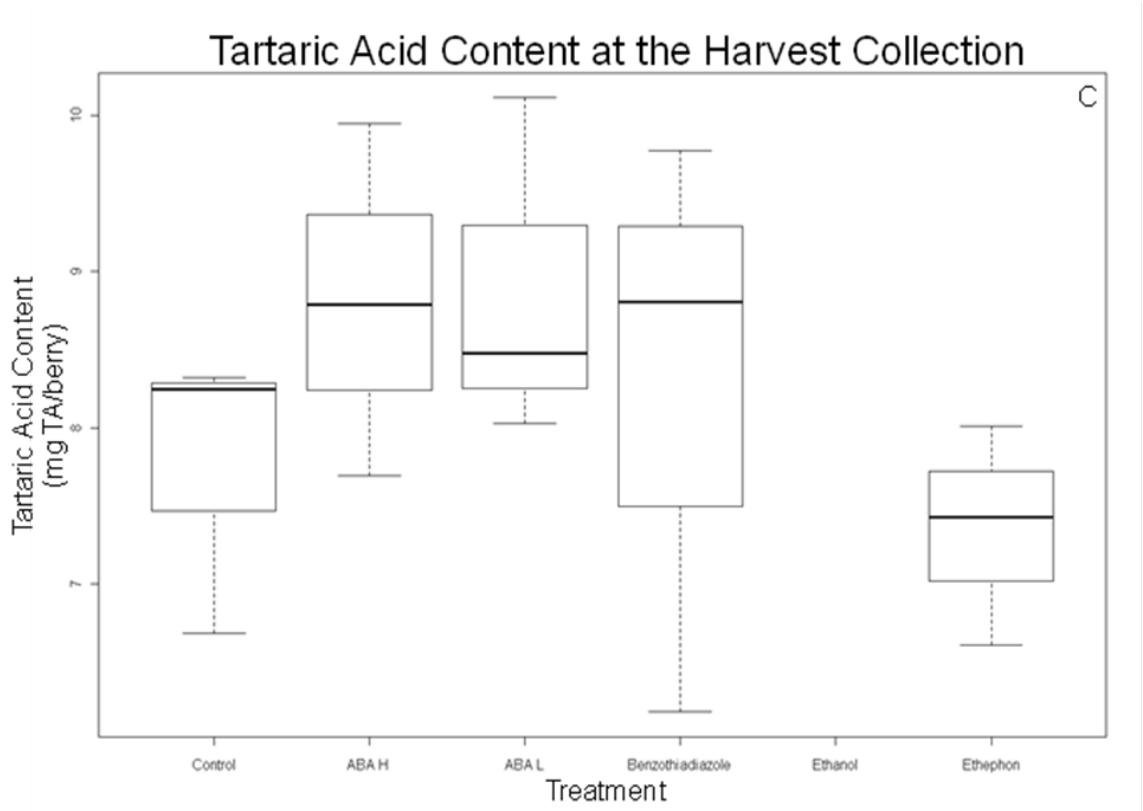

Tartaric Acid Content at the Second Collection
B
Tartaric Acid Content
(mg TA/berry)
6
8
10
12
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

Figure A.5.3


Tartaric Acid Content at the Harvest Collection
C
Tartaric Acid Content
(mg TA/berry)
10
9
8
7
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

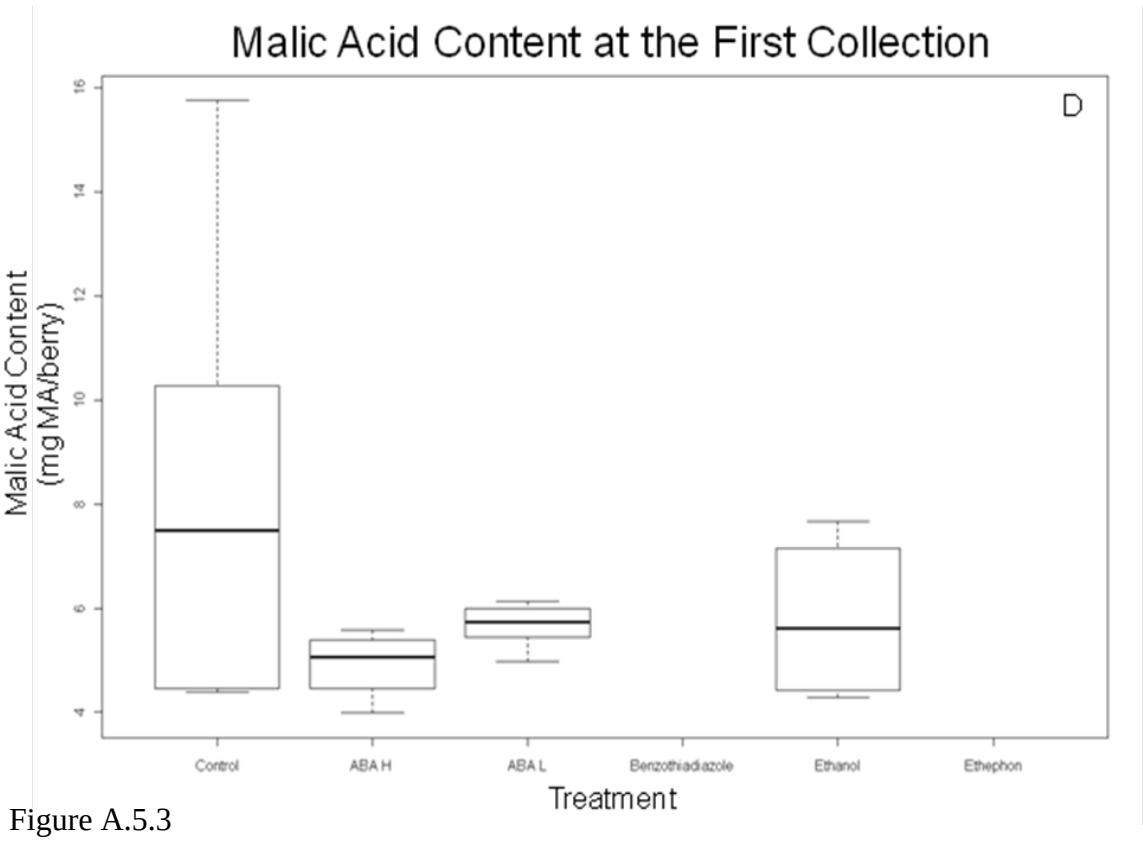
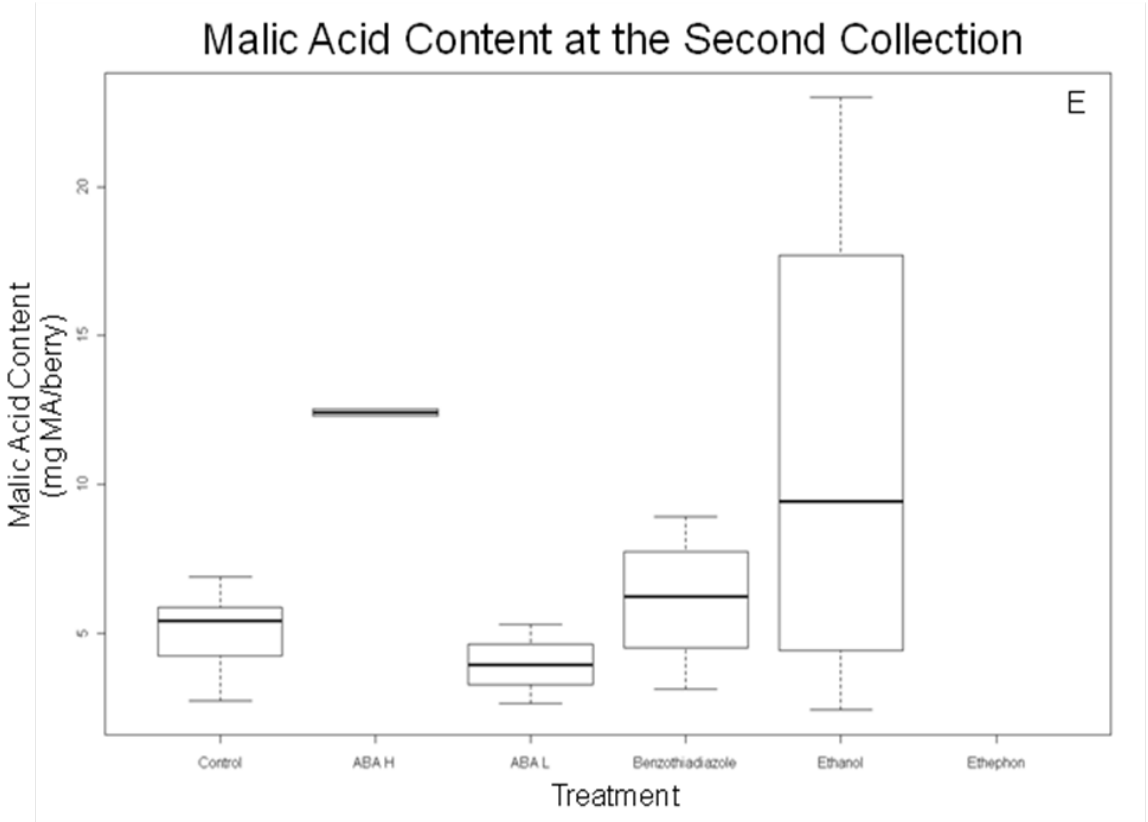

Malic Acid Content at the First Collection
D
Malic Acid Content
(mg MA/berry)
16
14
12
10
8
6
4
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

Figure A.5.3


Malic Acid Content at the Second Collection
E
Malic Acid Content
(mg MA/berry)
5
10
15
20
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

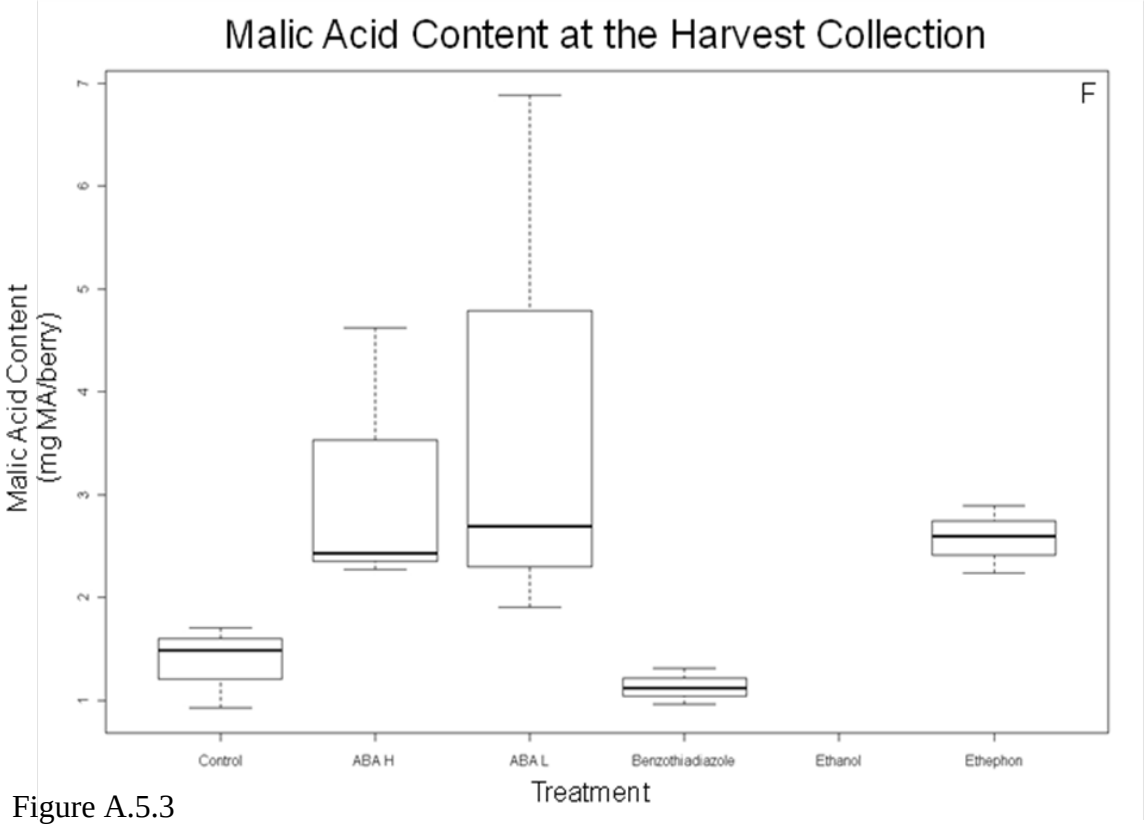
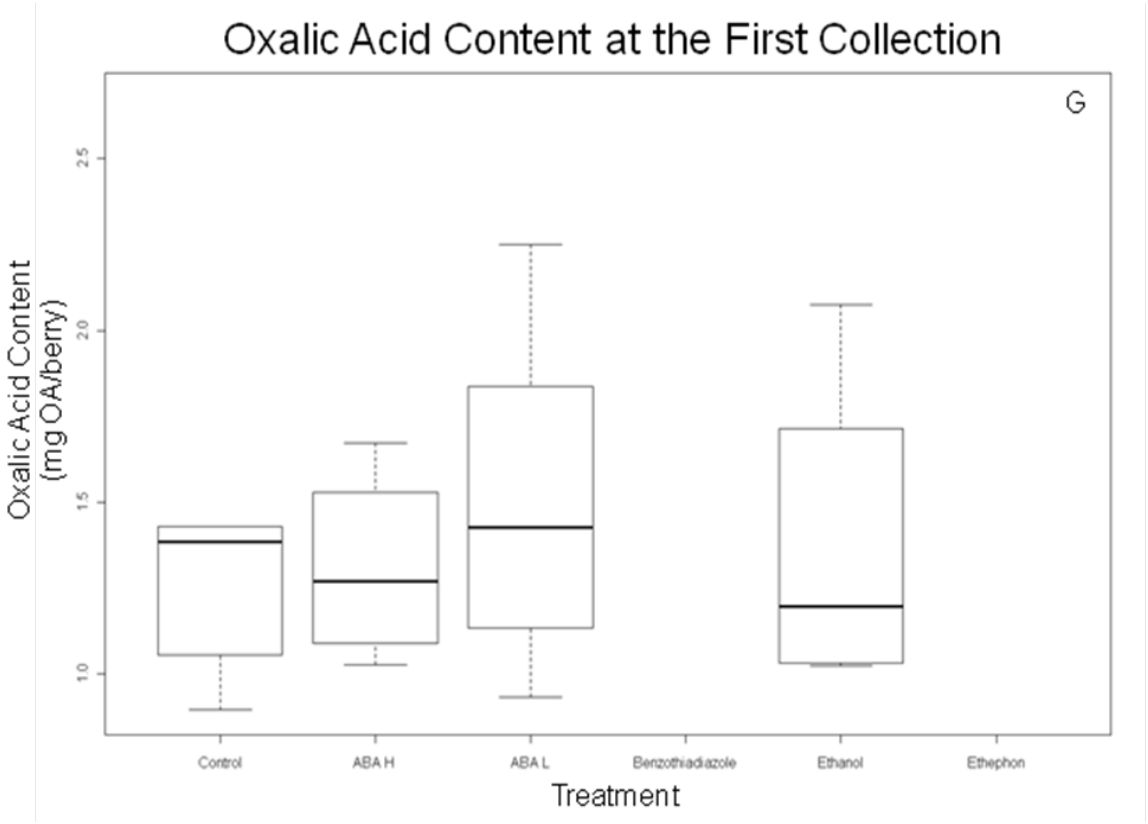

Malic Acid Content at the Harvest Collection
F
Malic Acid Content
(mg MA/berry)
1
2
3
4
5
6
7
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

Figure A.5.3


Oxalic Acid Content at the First Collection
G
Oxalic Acid Content (mg OA/berry)
1.0
1.5
2.0
2.5
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

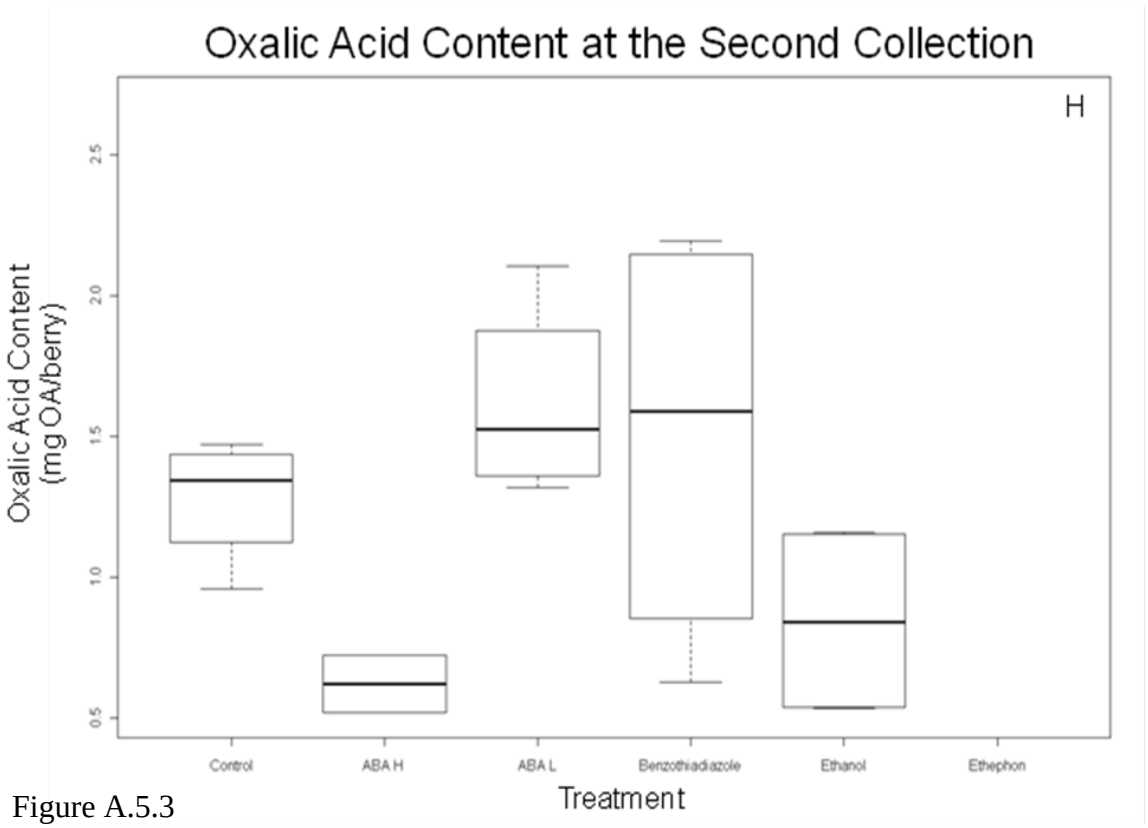
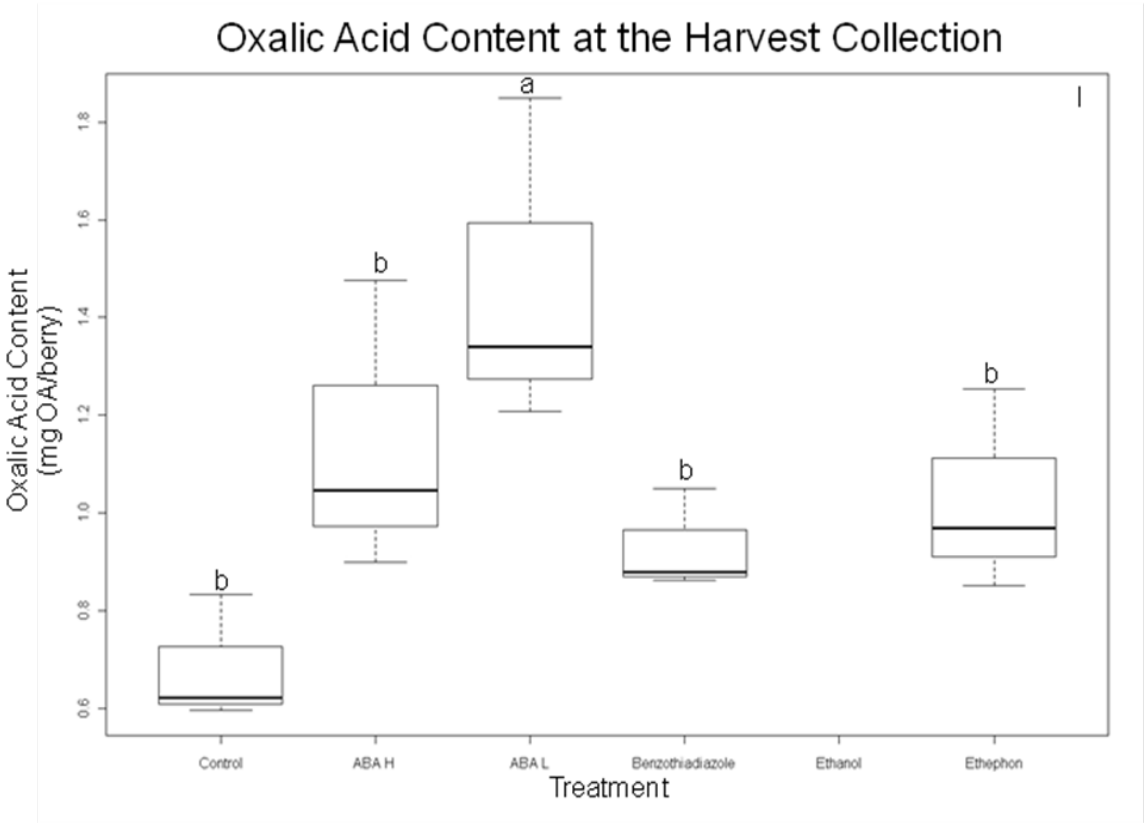

Oxalic Acid Content at the Second Collection
H
Oxalic Acid Content (mg OA/berry)
0.5
1.0
1.5
2.0
2.5
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

Figure A.5.3


Oxalic Acid Content at the Harvest Collection
I
Oxalic Acid Content
(mg OA/berry)
0.6
0.8
1.0
1.2
1.4
1.6
1.8
a
b
b
b
b
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

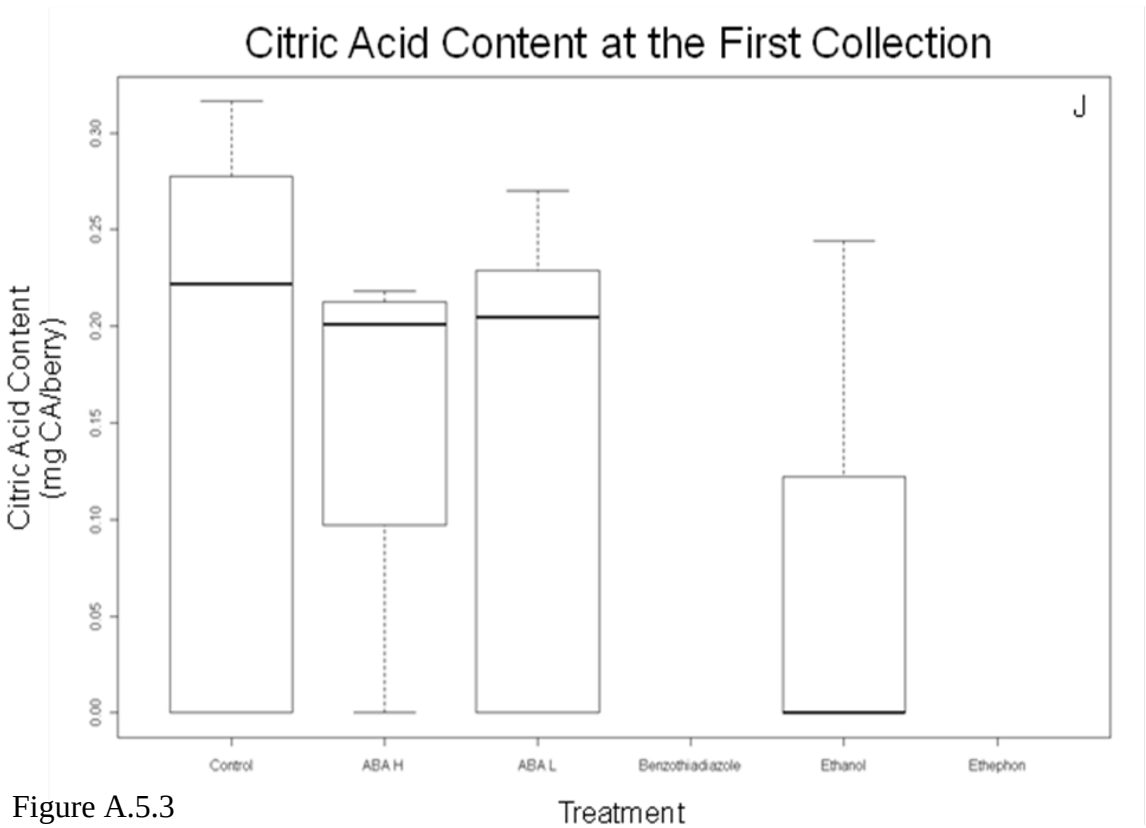
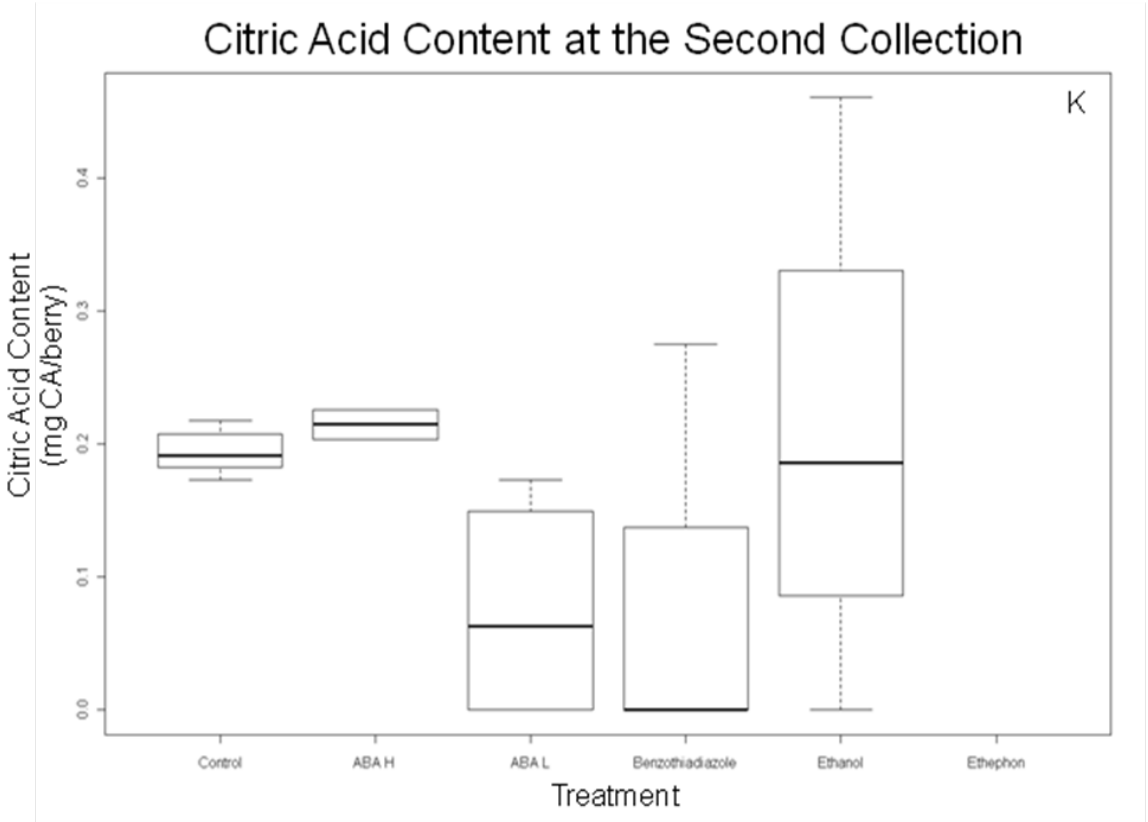

Citric Acid Content at the First Collection
J
Citric Acid Content
(mg CA/berry)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

Figure A.5.3


Citric Acid Content at the Second Collection
K
Citric Acid Content
(mg CA/berry)
0.0
0.1
0.2
0.3
0.4
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

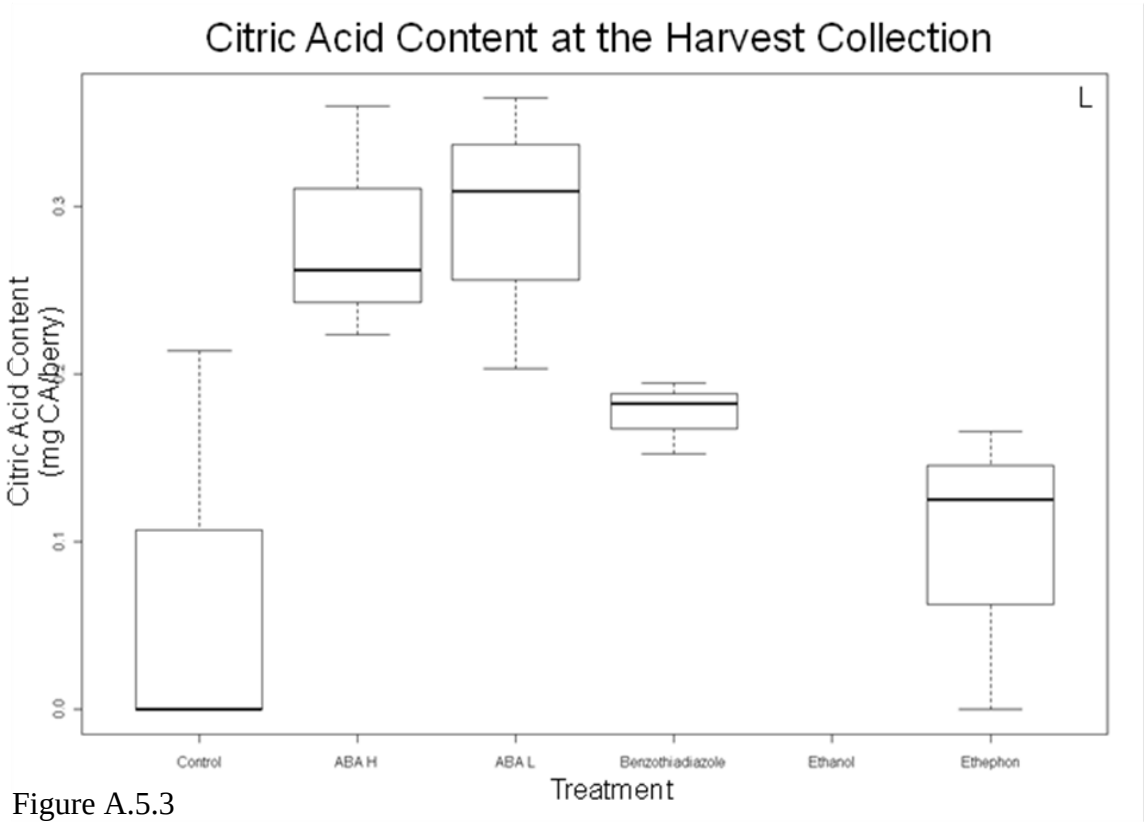
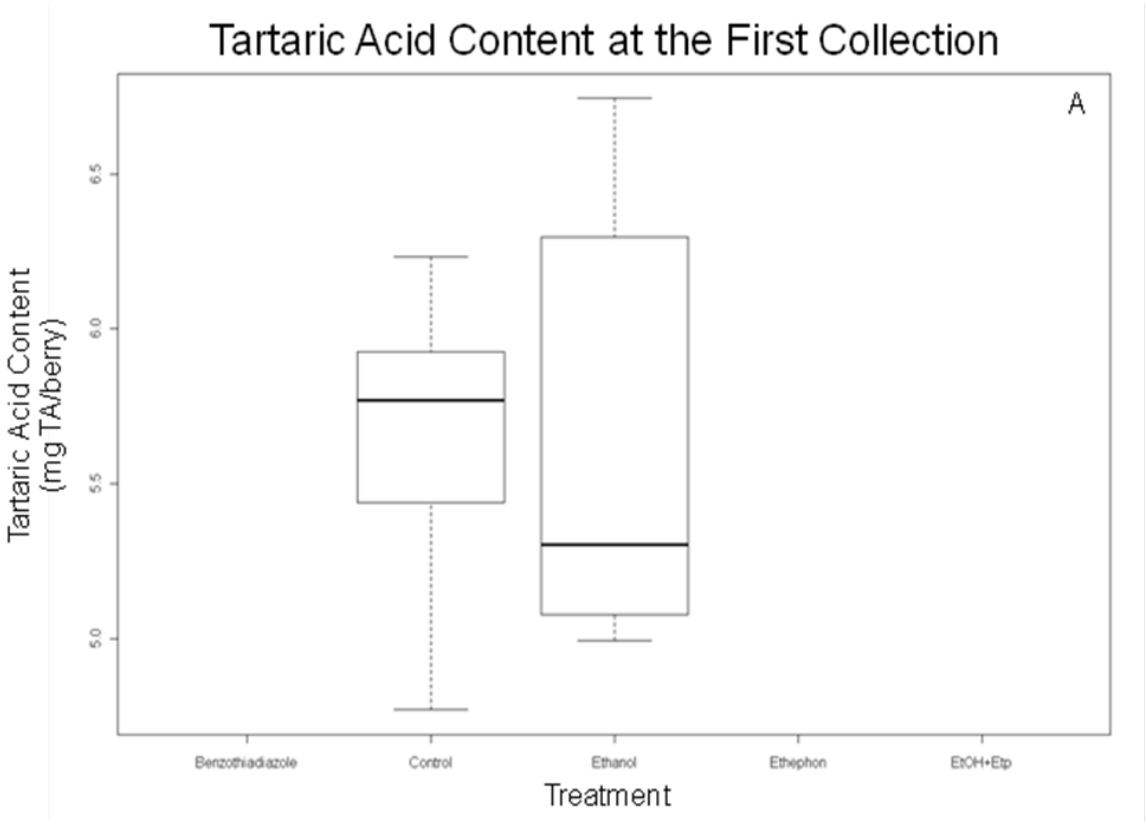

Citric Acid Content at the Harvest Collection
L
Citric Acid Content
(mg CA/berry)
0.0
0.1
0.2
0.3
Control
ABA H
ABA L
Benzothiadiazole
Ethanol
Ethephon
Treatment

Figure A.5.3

Figure A.5.3 The organic acid content of treatment Cabernet Franc grape clusters collected first on August 24th, second on September 1st, and at harvest on September 29th, 2009 with ABA high (ABA H), ABA low (ABA L), Benzothiadiazole, Control, Ethanol, and Ethephon treatments. (**A, B,C**), tartaric acid (TA) content for the first collection, second collection, and harvest collection, respectfully (**D, E, F**), malic acid (MA) content for the first, second, and harvest collections, respectfully (**G, H, I)**, oxalic acid (OA) content first, second, harvest collections, respectfully (**J, K, L**), citric acid (CA) content first, second, harvest collections, respectfully, content expressed as mg of the organic acid per berry. For oxalic acid content, significant difference in the mean content, $p \leq 0.05$, using Tukey's HSD (Honestly Significant Difference) test was denoted using lowercase letters. For each treatment, the heavy horizontal black line represents mean content, the box contains the upper and lower quartiles, and the bars with caps represents either the minimum/maximum or 1.5 times the interquartile range, whichever is smaller.


Tartaric Acid Content at the First Collection
A
Tartaric Acid Content
(mg TA/berry)
6.5
6.0
5.5
5.0
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

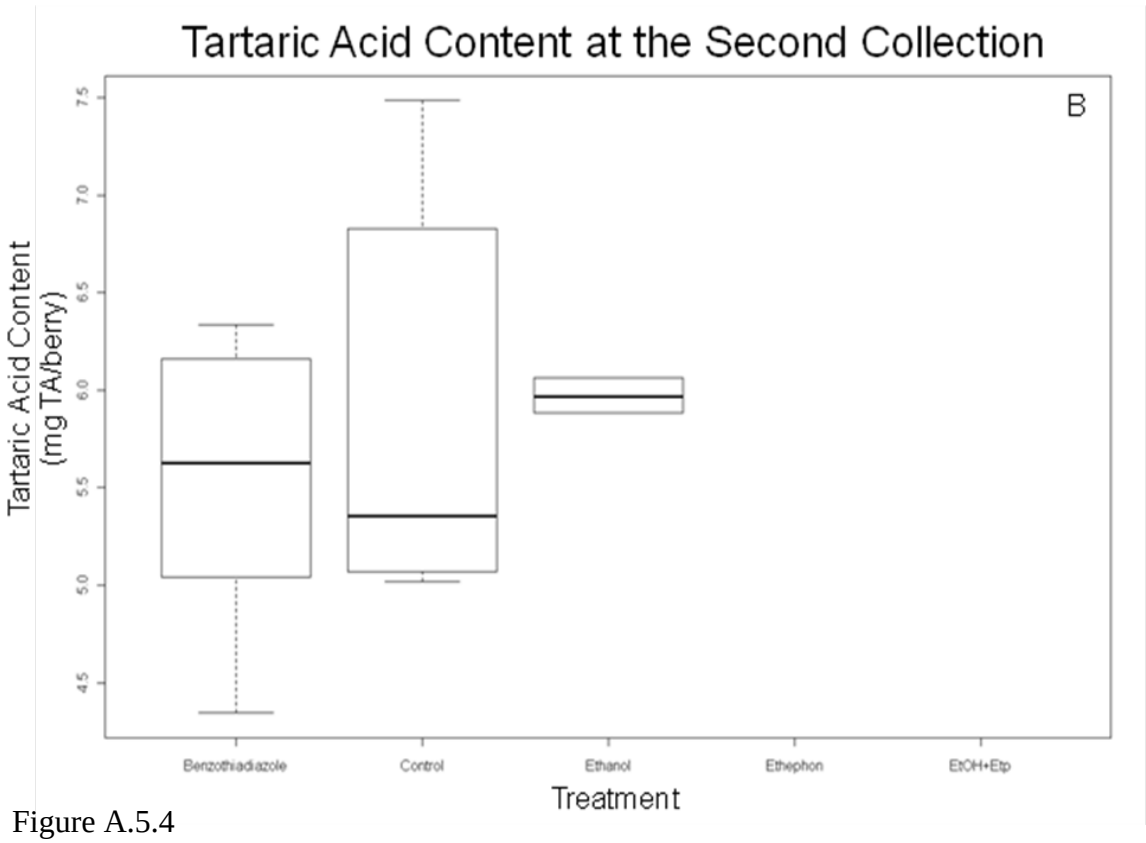
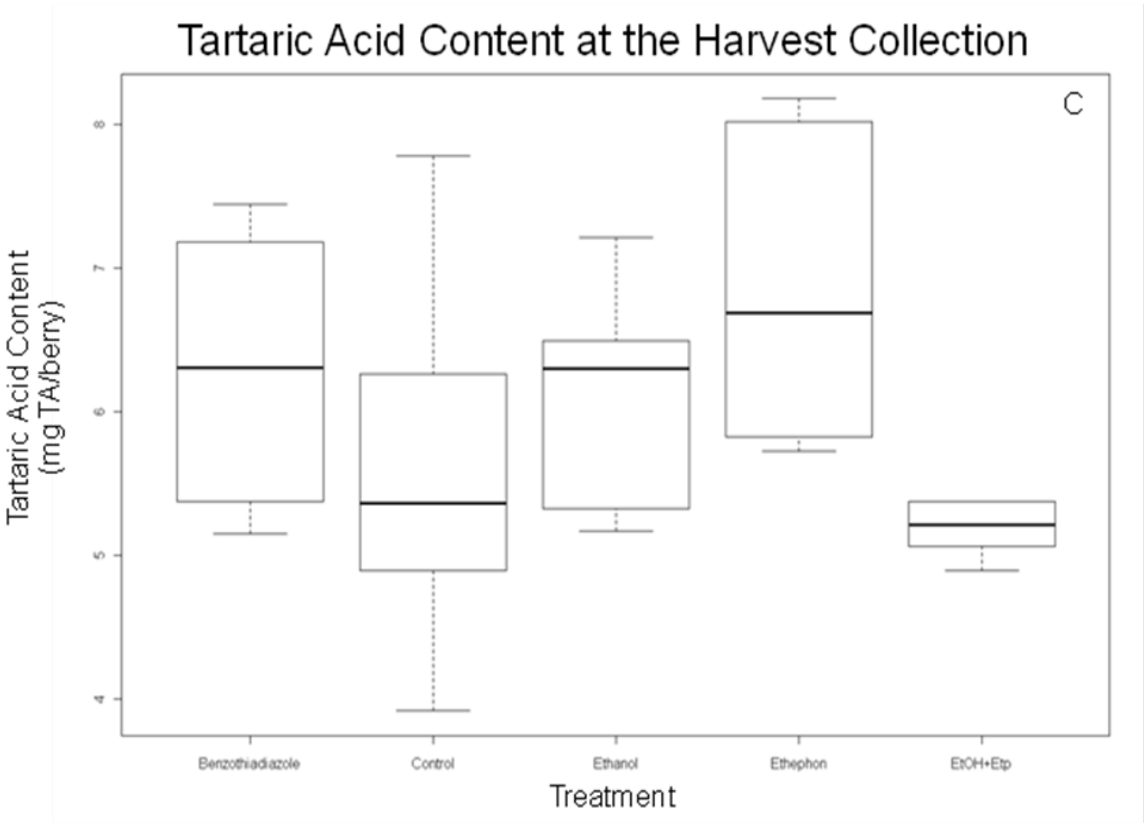

Tartaric Acid Content at the Second Collection
B
Tartaric Acid Content
(mg TA/berry)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

Figure A.5.4


Tartaric Acid Content at the Harvest Collection
C
Tartaric Acid Content
(mg TA/berry)
4
5
6
7
8
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

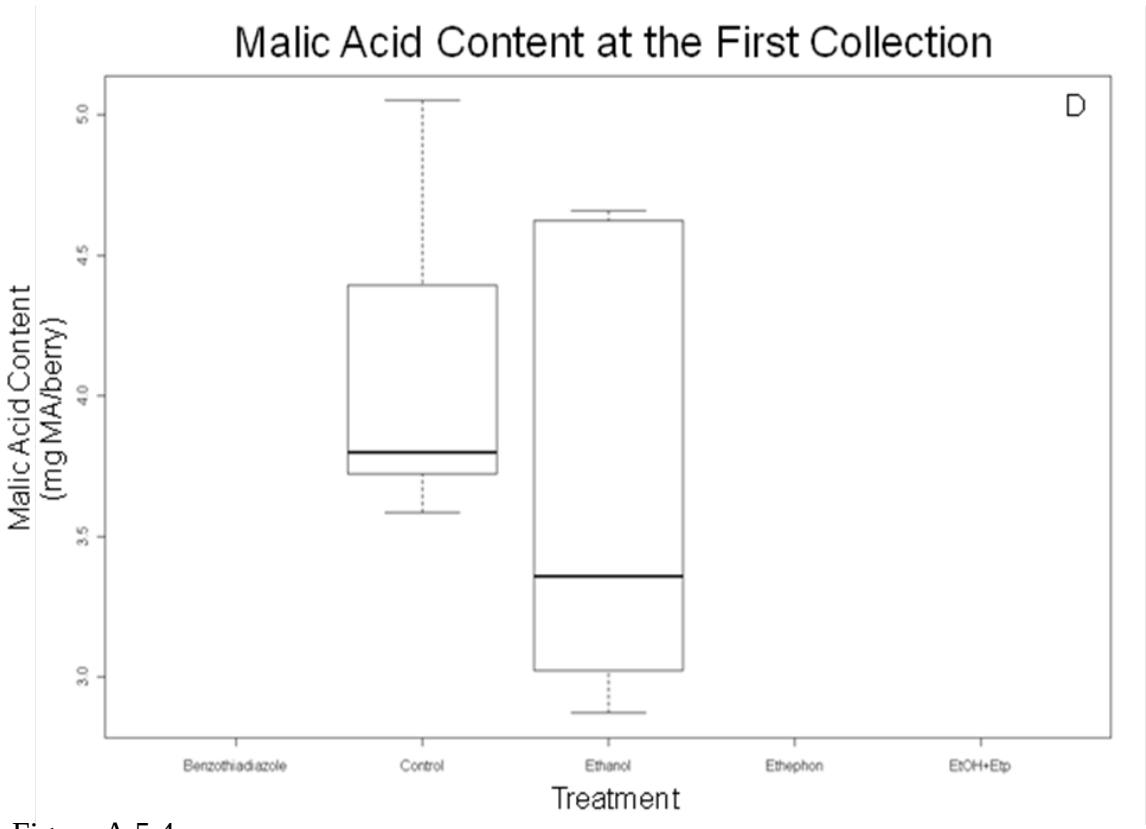
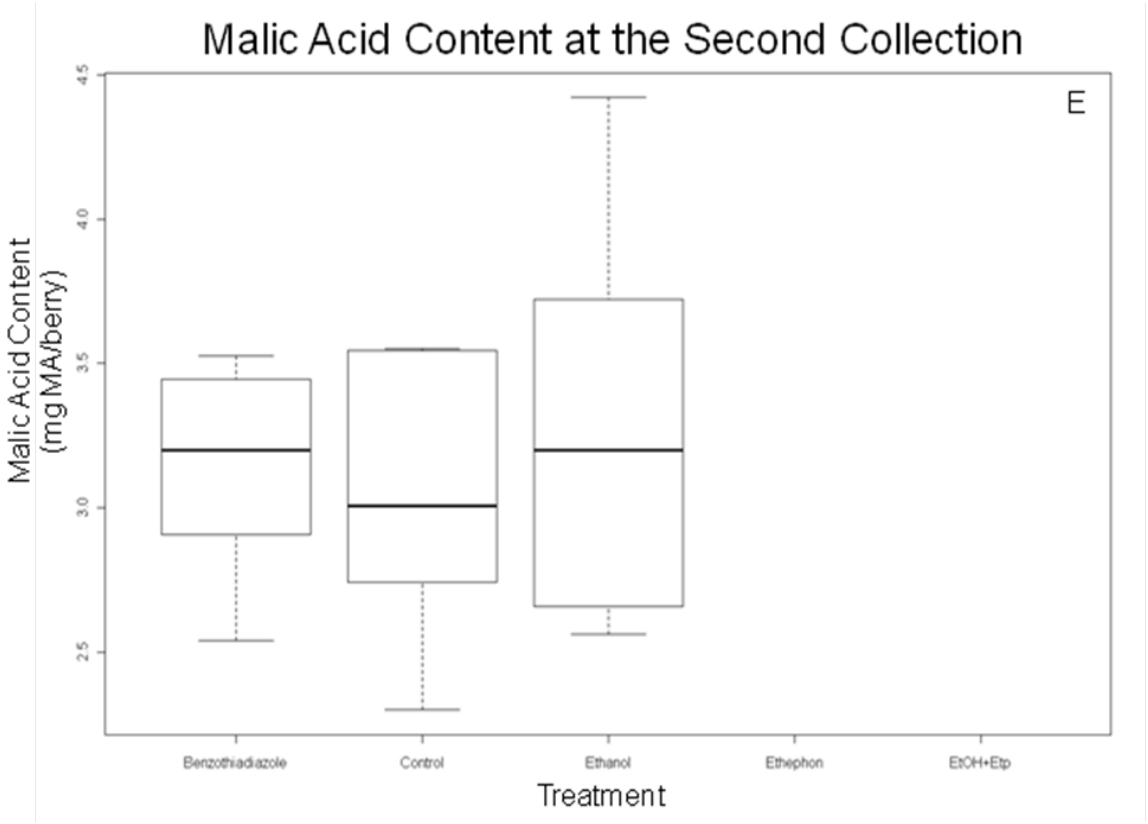

Malic Acid Content at the First Collection
D
Malic Acid Content
(mg MA/berry)
3.0
3.5
4.0
4.5
5.0
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

Figure A.5.4


Malic Acid Content at the Second Collection
E
Malic Acid Content
(mg MA/berry)
45
40
35
30
25
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

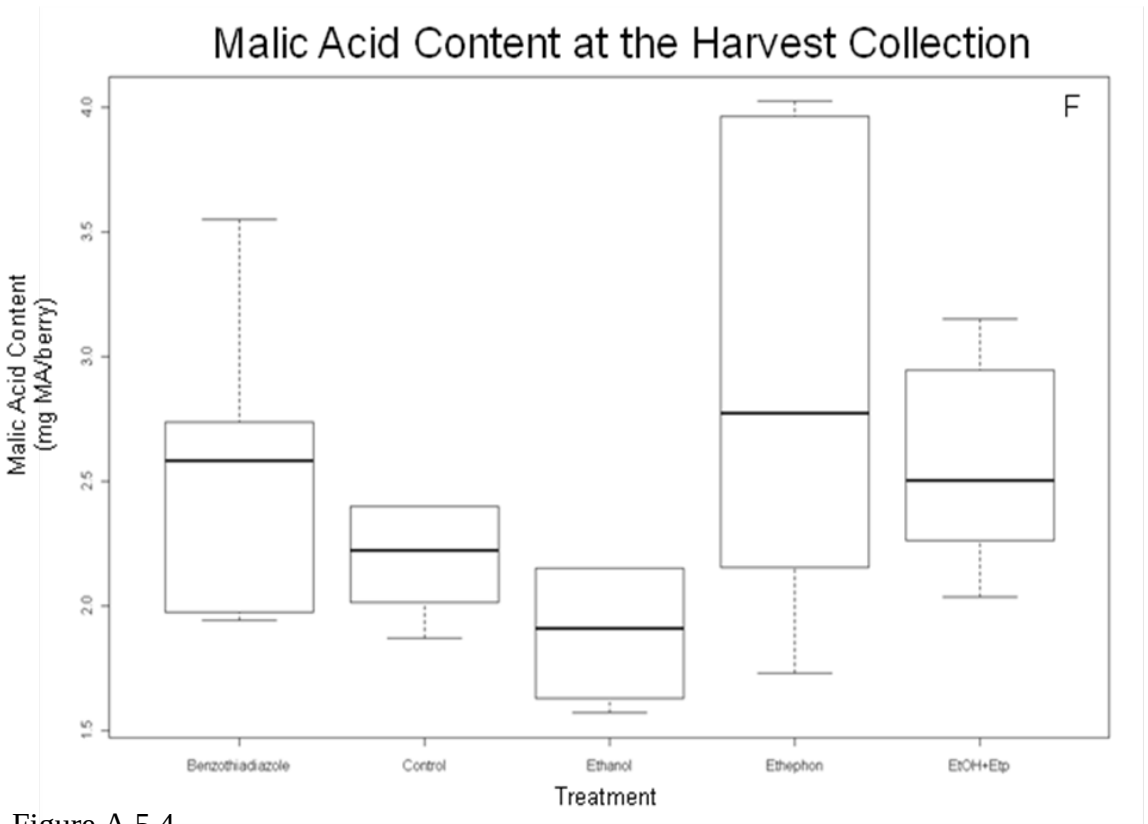
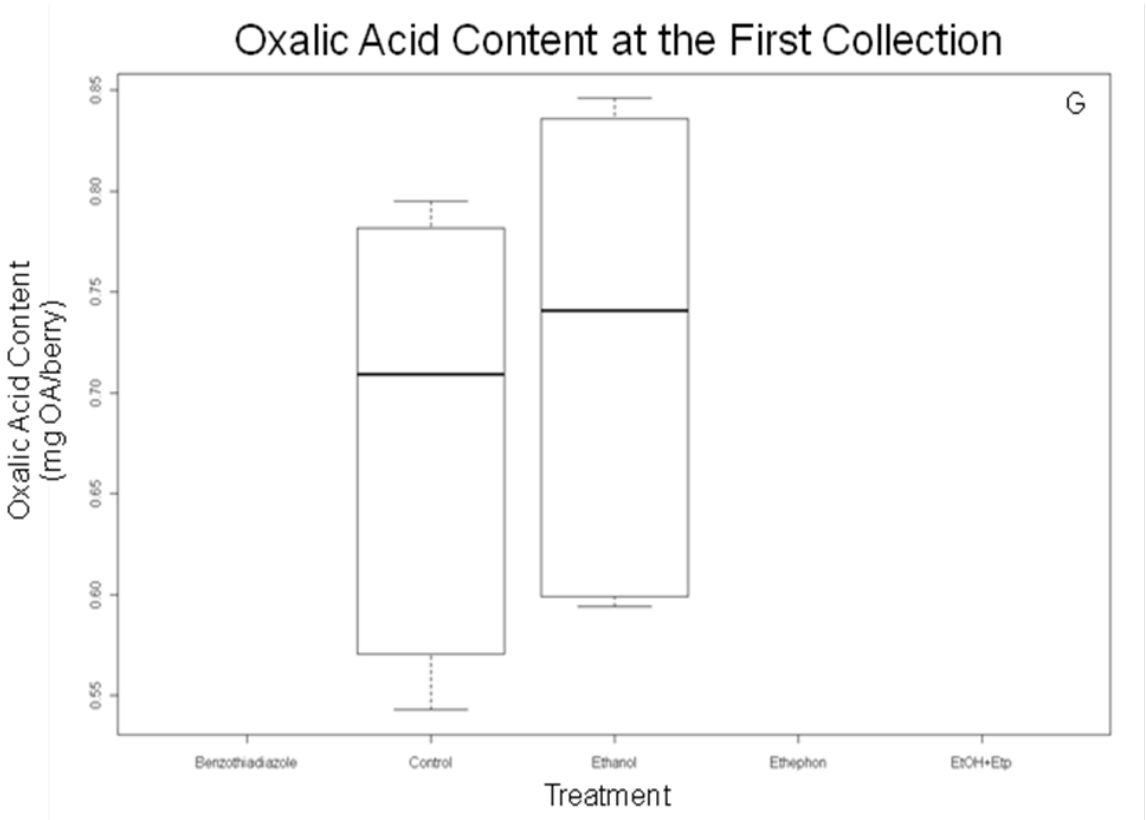

Malic Acid Content at the Harvest Collection
F
Malic Acid Content
(mg MA/berry)
40
35
30
25
20
15
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

Figure A.5.4


Oxalic Acid Content at the First Collection
G
Oxalic Acid Content
(mg OA/berry)
0.55
0.60
0.65
0.70
0.75
0.80
0.85
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

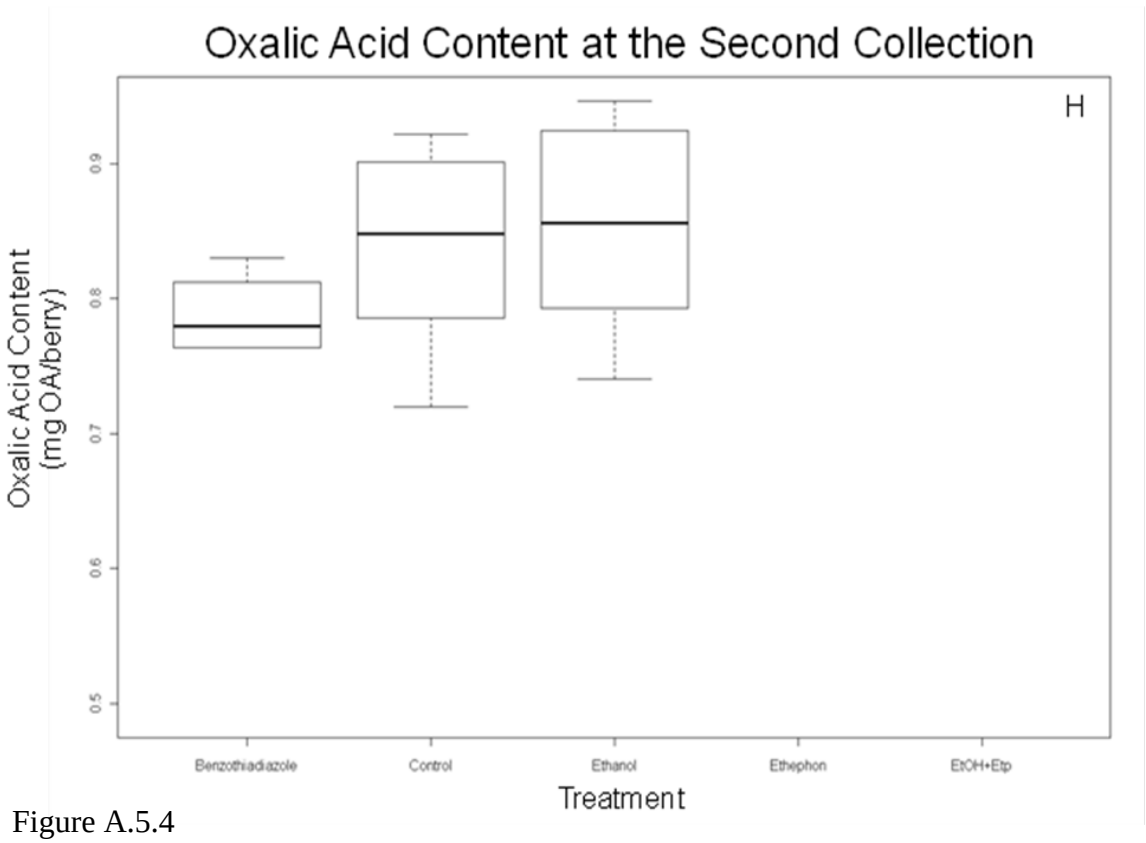
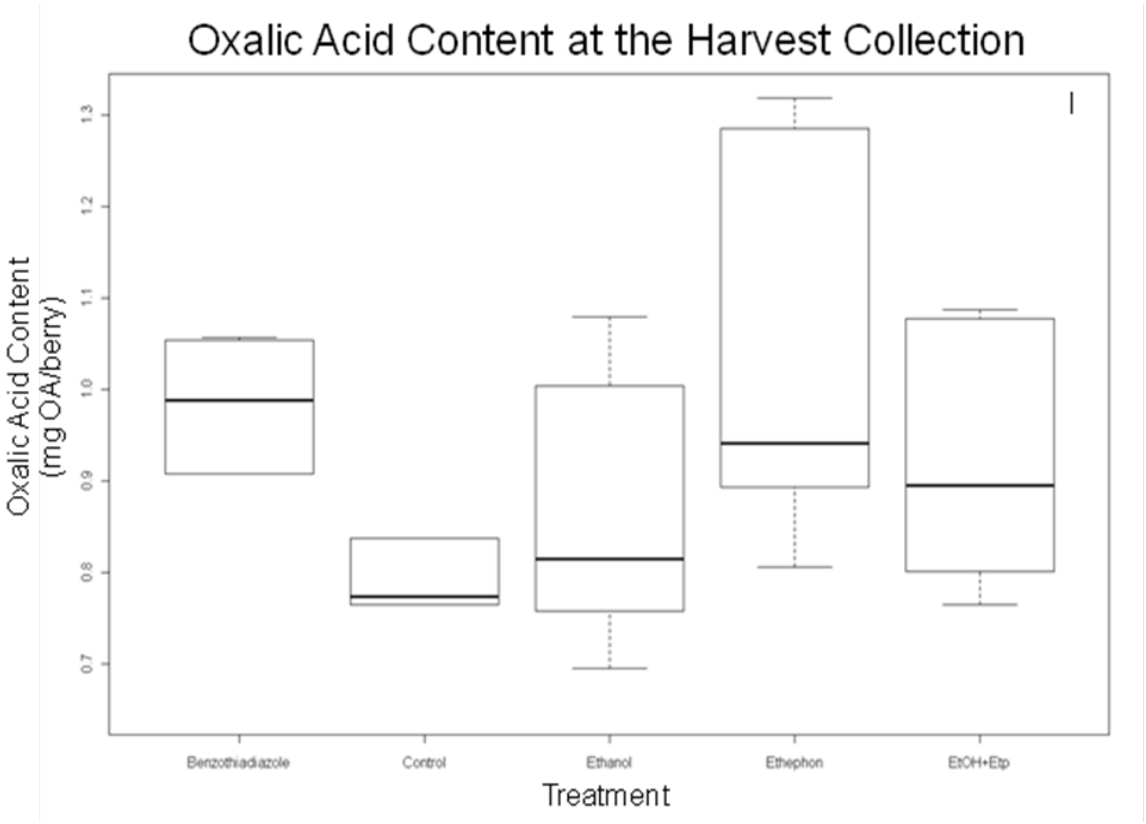

Oxalic Acid Content at the Second Collection
H
Oxalic Acid Content
(mg OA/berry)
0.5
0.6
0.7
0.8
0.9
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

Figure A.5.4


Oxalic Acid Content at the Harvest Collection
I
Oxalic Acid Content
(mg OA/berry)
0.7
0.8
0.9
1.0
1.1
1.2
1.3
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

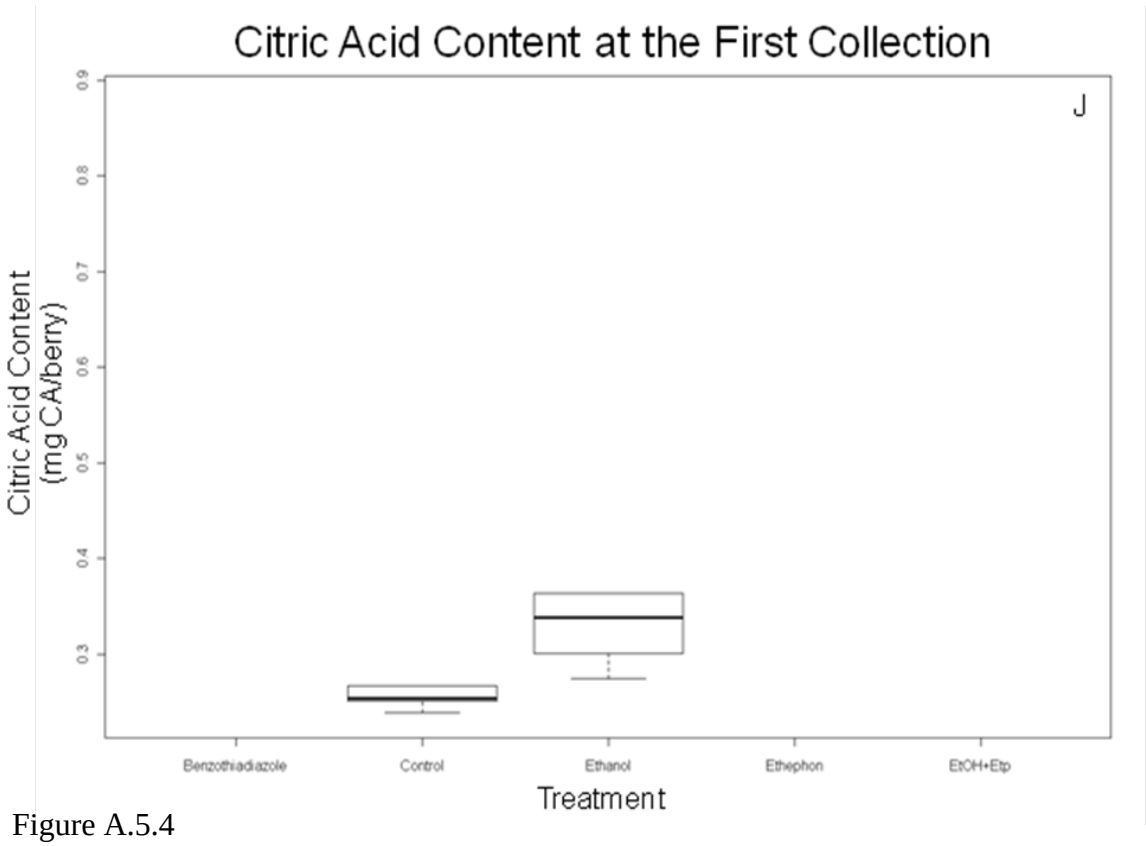
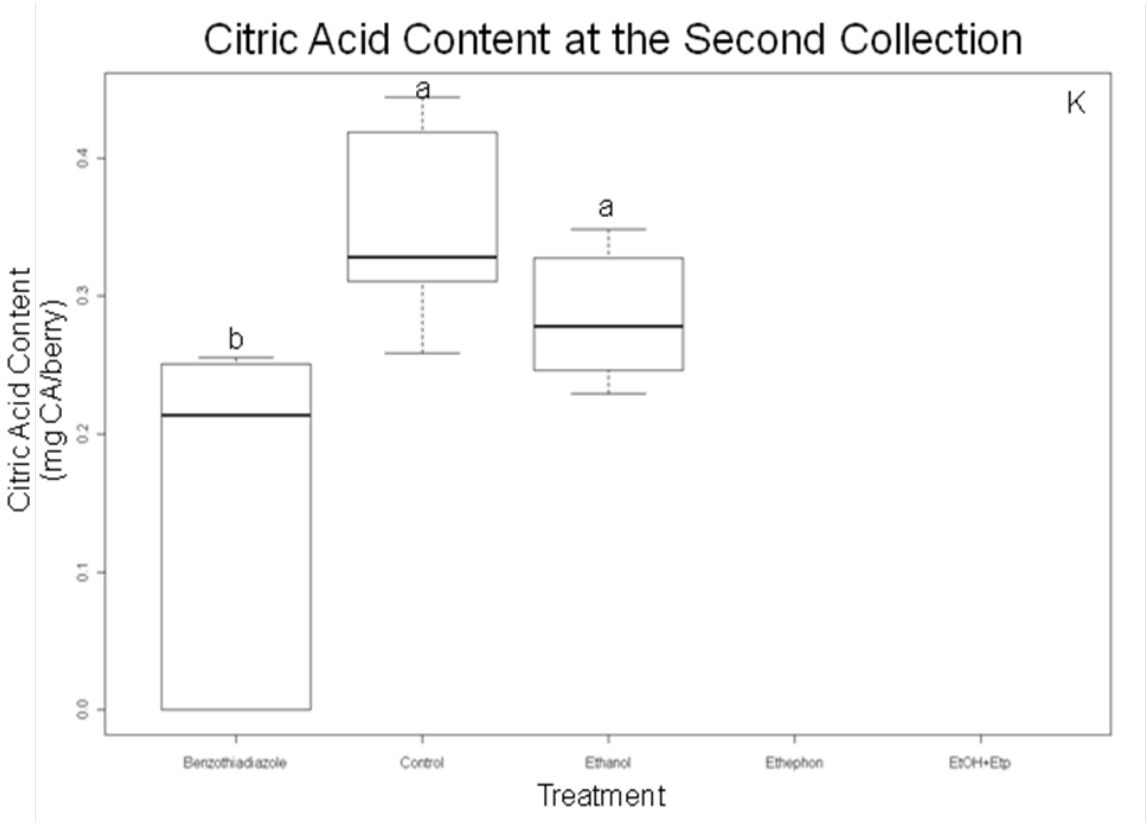

Citric Acid Content at the First Collection
J
Citric Acid Content
(mg CA/berry)
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

Figure A.5.4


Citric Acid Content at the Second Collection
K
a
a
b
Citric Acid Content (mg CA/berry)
0.0
0.1
0.2
0.3
0.4
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

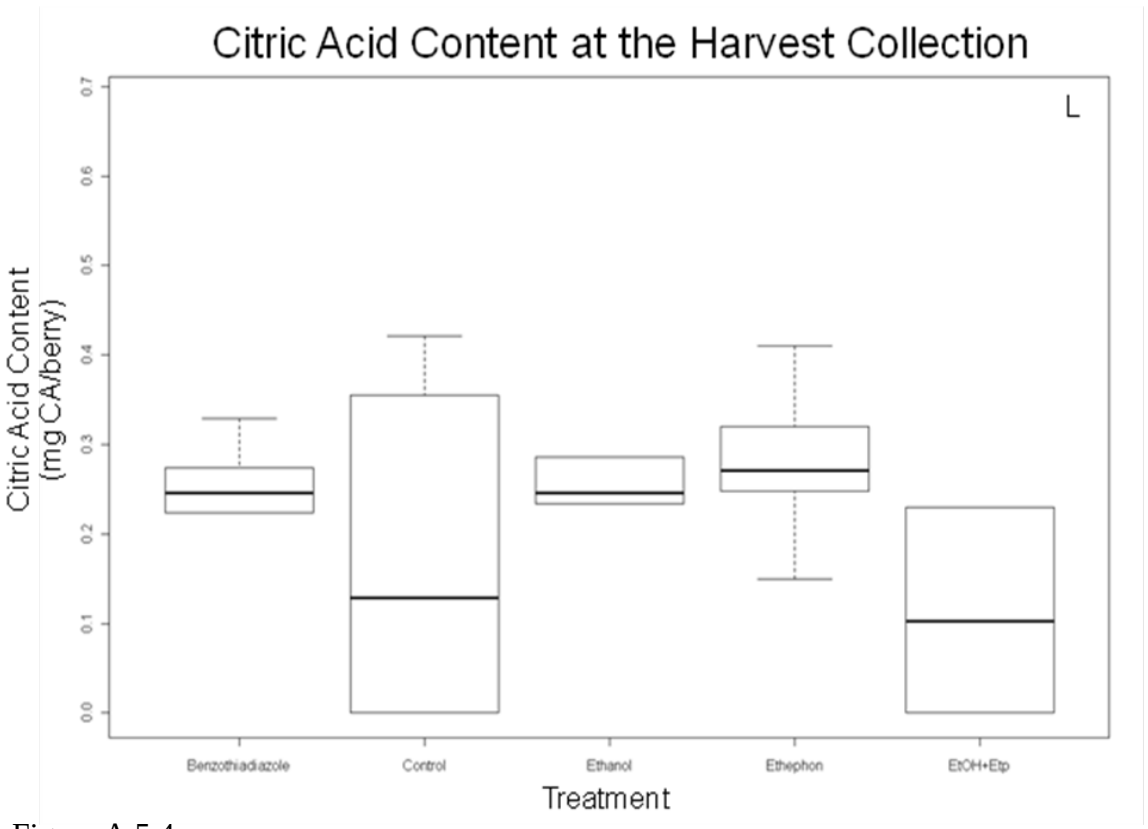

Citric Acid Content at the Harvest Collection
L
Citric Acid Content (mg CA/berry)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Benzothiadiazole
Control
Ethanol
Ethephon
EtOH+Etp
Treatment

Figure A.5.4

Figure A.5.4 The organic acid content of treatment Cabernet Franc grape clusters collected first on August 20th, second on August 27th , and at harvest on September 14th, 2010 with Benzothiadiazole, Control, Ethanol, Ethephon, and Ethanol with Ethephon (EtOH+Etp) treatments. (**A, B,C**), tartaric acid (TA) content for the first collection, second collection, and harvest collection, respectfully (**D, E, F**), malic acid (MA) content for the first, second, and harvest collections, respectfully (**G, H, I)**, oxalic acid (OA) content first, second, harvest collections, respectfully (**J, K, L**), citric acid (CA) content first, second, harvest collections, respectfully, content expressed as mg of the organic acid per berry. For citric acid content, significant difference in the mean content, $p \leq 0.05$, using Tukey's HSD (Honestly Significant Difference) test was denoted using lowercase letters. For each treatment, the heavy horizontal black line represents mean content, the box contains the upper and lower quartiles, and the bars with caps represents either the minimum/maximum or 1.5 times the interquartile range, whichever is smaller.

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VITA

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